Gain-of-Function Effects of Mutant p53 Explored Using a Three-Dimensional Culture Model of Breast Cancer

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ABSTRACT

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p53 is the most frequent target for mutation in human tumors and mutation at this locus is a common and early event in breast carcinogenesis. Breast tumors with mutated p53 often contain abundant levels of this mutant protein, which has been postulated to actively contribute to tumorigenesis by acquiring pro-oncogenic ("gain-of-function") properties. To elucidate how mutant p53 might contribute to mammary carcinogenesis, we employed a three-dimensional (3D) culture model of breast cancer. When placed in a laminin-rich extracellular matrix, non-malignant mammary epithelial cells form structures highly reminiscent for many aspects of acinar structures found *in vivo*. On the other hand, breast cancer cells, when placed in the same environment, form highly disorganized and sometimes invasive structures. Modulation of critical oncogenic signaling pathways has been shown to phenotypically revert breast cancer cells to a more acinar-like morphology.

We examined the role of mutant p53 in this context by generating stable, regulatable p53 shRNA derivatives of mammary carcinoma cell lines to deplete endogenous mutant p53. We demonstrated that, depending on the cellular context, mutant p53 depletion is sufficient to significantly reduce invasion or in some cases actually induce a phenotypic reversion to more acinar-like structures in breast cancer cells grown in 3D culture. Additionally, using stable overexpression of a panel of

tumor-derived p53 mutants in non-malignant mammary epithelial cells, we found that mutant p53 is sufficient to disrupt normal acinar morphogenesis. Genome-wide expression analysis identified the mevalonate pathway as significantly upregulated by mutant p53. Statins and sterol biosynthesis intermediates revealed that this pathway is both necessary and sufficient for the phenotypic effects of mutant p53 on mammary tissue architecture. We then showed that mutant p53 associates with sterol gene promoters at least partly via SREBP transcription factors. Finally, p53 mutation correlates with highly expressed mevalonate pathway genes in human breast tumors and elevated expression of the mevalonate pathway correlates with a poor prognosis in breast cancer.

We also queried a number of pathways/proteins that had previously been implicated in breast cancer and shown to be sufficient to bring about a phenotypic reversion in 3D culture to search for additional mechanisms by which mutant p53 might contribute to mammary carcinogenesis. Using this approach, we identified integrin β4 as a novel target of mutant p53 in breast cancer cells and demonstrated that stable knockdown of integrin β4 is sufficient to dramatically reduce invasive processes in breast cancer cells grown in 3D culture. We also show that mutant p53 associates with the promoter of *ITGB4*, the gene encoding integrin β4. Finally, we demonstrated that inhibition of NF-κB, a downstream mediator of integrin β4signaling, can mimic the phenotypic effects of mutant p53 depletion.

These findings contribute to our understanding of breast carcinogenesis and may offer novel prognostic indicators and therapeutic targets for tumors bearing mutations in p53.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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DEDICATION

I dedicate this thesis to my mother, Marilynne Freed.

From an early age, she instilled in me a thirst for knowledge, a desire to understand the way the world works. Part of my motivation to pursue cancer research is surely an attempt to quench this thirst, but even more so it stems from her battle with pancreatic cancer. Although this work is a paltry gift, I dedicate it to her memory.

Chapter 1

Introduction

The *TP53* gene, which resides on chromosome 17p13.1 and encodes the p53 protein, is the most frequent target for mutation in human cancer, with greater than half of all tumors exhibiting mutation at this locus (Petitjean et al., 2007b; Vogelstein et al., 2000).

The discovery of the p53 "proto-oncogene"

The p53 protein was first identified in a complex with the simian virus 40 (SV40) large T-antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). It was subsequently demonstrated that many tumors produce abundant levels of this 53 kDa protein, a phenomenon that was not observed in normal tissue, suggesting that this tumor-associated protein might act as a cellular oncogene (DeLeo et al., 1979; Rotter, 1983). This notion was reinforced when overexpression of newly cloned p53 cDNAs were shown to cooperate with oncogenic Ras to transform primary cells in culture (Eliyahu et al., 1984; Parada et al., 1984). Furthermore, overexpression of p53 was demonstrated to increase tumorigenicity in otherwise p53-null cells (Wolf et al., 1984). Thus, throughout the first decade after its discovery, p53 was generally acknowledged as a proto-oncogene (Levine and Oren, 2009).

However, multiple early findings called into question the role of p53 as a cellular oncogene. For example, the murine *Trp53* gene was shown to be inactivated by retroviral insertions in several tumor models (Ben David et al., 1988; Wolf and Rotter, 1984). To resolve discrepant findings regarding the ability of p53 to transform primary cells, several groups compared the sequences of cloned p53 cDNAs only to find out that no two clones were identical in sequence (Levine and Oren, 2009). It was soon recognized that these early experiments

demonstrating that p53 overexpression could transform cells and promote *in vivo* tumor growth, were actually performed with mutated versions of p53 which had been isolated from tumor cells (Hinds et al., 1989; Hinds et al., 1990; Levine and Oren, 2009). Consequently, while early experiments were originally thought to describe the function of wild-type p53, they were in fact detailing the role of mutant p53 in tumor biology (the importance of which will become apparent below).

In 1989, two seminal findings overturned the widely accepted notion that p53 acts as a proto-oncogene. Vogelstein and colleagues, investigated genetic alterations in colorectal carcinomas and demonstrated that greater than 50% of these tumors exhibit loss-of-heterozygosity (LOH), a hallmark of tumor suppressor genes, at the *TP53* locus (most commonly: mutation of one *TP53* allele and deletion of the corresponding *TP53* allele) (Baker et al., 1989). Levine and colleagues demonstrated that overexpression of wild-type p53 was actually sufficient to suppress oncogenic transformation (Finlay et al., 1989). These and subsequent findings firmly established p53 as a tumor suppressor gene. However, one should never discount seemingly contradictory results and it turns out we can still gain deep insight into the role of p53 in tumor biology from these early "artifacts" studying mutant p53.

p53 and tumor suppression

Soon after this paradigm shift took place in the p53 field, a number of studies served to confirm the role of wild-type p53 as a tumor suppressor and establish this protein as one of the most important players in cancer biology. In the late 1960s, a number of extremely cancer-prone families were identified in the United States and Europe (Li and Fraumeni, 1969a, b). This familial cancer syndrome came to be known as Li-Fraumeni syndrome (LFS), a rare autosomal-

dominant disorder which predisposes to breast cancer, sarcomas and other neoplasms. LFS was later shown to be caused by germline mutations in *TP53* (Malkin et al., 1990; Srivastava et al., 1990). A mouse model was soon developed in which p53 was disrupted by homologous recombination to explore the role of this protein in cancer. While p53-/- mice were developmentally normal, they were extremely cancer-prone (Attardi and Jacks, 1999; Donehower et al., 1992). Mice devoid of p53 exhibited an extreme susceptibility to multiple tumor types, primarily lymphomas and sarcomas, with three out of every four p53-/- mice having developed at least one obvious neoplasm by six months of age. In contrast, wild-type littermates failed to develop any tumors by nine months of age (Donehower et al., 1992). In addition to the early findings by the Vogelstein group, myriad studies have now confirmed that *TP53* mutations are not isolated to colorectal cancer, but are present in greater than 50% of all human tumors (Petitjean et al., 2007b; Vogelstein et al., 2000). This has led some to speculate that p53 mutations are the most common genetic event in all of human cancer (Levine and Oren, 2009).

In fact, it has been hypothesized that p53 function is compromised in most, if not all, human tumors (Polager and Ginsberg, 2009). As mentioned above, most tumors exhibit mutation of p53; however, in tumors which retain wild-type p53, its activity can be attenuated by a number of other mechanisms. For example, many DNA tumor viruses encode proteins which can inactivate p53; SV40 large T-antigen, adenovirus E1B-55 kDa protein and the E6 oncoprotein of human papilloma virus (HPV) types 16 and 18 all bind to p53 and inactivate its function (Levine and Oren, 2009). The biological relevance of which is highlighted by the fact that HPV types 16 and 18 have been implicated in cervical carcinogenesis (Ferenczy and Franco, 2002). Another mechanism by which tumors inactivate p53 is through the upregulation or activation of negative regulators of p53. Mdm2, an E3 ubiquitin ligase, is the major negative

regulator of p53 and serves to keep p53 levels in check under unstressed conditions (Poyurovsky and Prives, 2006). Additionally, a homolog of Mdm2, MdmX (also known as Mdm4), also serves as a negative regulator of p53. Not surprisingly, both Mdm2 and MdmX are overexpressed in a variety of neoplasms (Marine et al., 2006).

Importantly, not only has p53 been demonstrated to be inactivated in most tumors, but a number of *in vivo* studies have demonstrated that reactivation of wild-type p53 in p53 null or p53 mutant tumors is sufficient to lead to tumor stasis/regression (Christophorou et al., 2005; Christophorou et al., 2006; Kenzelmann Broz and Attardi, 2010; Xue et al., 2007). This observation has paved the way for multiple approaches to activate wild-type p53 in tumor cells.

p53 and therapeutic approaches

Three of the more exciting approaches include small molecules that inhibit the interaction between p53 and its negative regulators (Mdm2 and/or MdmX), gene therapy to deliver wild-type p53 to tumors and small molecules to re-activate mutant p53 to a wild-type conformation. A number of other strategies are currently in development which also seek to target the p53 pathway or take advantage of p53 mutation in a large proportion of tumors.

While targeted therapeutics to disrupt the p53:Mdm2 complex are still in development, it is interesting to note that many clinically relevant chemotherapeutics lead to stabilization of p53. They do this primarily through induction DNA damage, which activates such pathways as the ATM/Chk2 pathway, phosphorylating p53 and releasing it from the negative feedback loop. In terms of the targeted therapies to prevent the p53:Mdm2 interaction, this approach is typified by the Nutlins (particularly Nutlin-3a) and RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis) (Issaeva et al., 2004; Vassilev et al., 2004). These small molecules bind directly to

Mdm2 or p53, respectively, breaking the interaction between p53 and Mdm2, thus stabilizing the p53 protein.

While gene therapy may seem like one of the most straightforward approaches given the early success of this approach in preclinical models, this has been one of the slowest to reach the clinic. Currently there are two p53 based gene therapies approved for clinical use in China and at least two in clinical trials in the United States (Levine and Oren, 2009). Both approaches involve adenoviral-based delivery of wild-type p53. The first is a replication-deficient adenovirus containing human p53 cDNA (Wolf et al., 2004) and the second is a replication-competent adenovirus carrying human p53 cDNA, however this adenovirus has the E1B-region deleted so it can only replicate in and kill p53-deficient cells (Heise et al., 1997).

The third approach involves "re-activating" mutant p53 to restore wild-type p53 function in mutant p53 expressing cells, allowing p53 to carry out its tumor suppressive functions in cancer cells (Brown et al., 2011). One of the first compounds reported to "re-activate" mutant p53 was CP31398, a small molecule that allowed mutant p53 expressing cells to induce the expression of canonical p53 target genes, drive expression from a p53 reporter construct and even impair tumor growth (Foster et al., 1999). Despite this promising start, it was later discovered that instead of binding directly to mutant p53, CP31398 intercalated into DNA (Rippin et al., 2002). However, this setback has not dismayed the many groups looking to identify compounds that restore p53 activity in mutant p53 tumors. While many groups are pursuing this approach, by far the most successful so far has been PRIMA-1 (also known as APR-246) has been shown to re-activate missense mutants of p53 to regain at least some functions of wild-type p53 and thus halt tumor growth and is currently in a Phase I clinical trial (Bykov et al., 2002; Wiman, 2010).

However, PRIMA-1 has also been shown to exert p53-independent activities potentially complicating its use in the clinic (Brown et al., 2011).

While the above mentioned compounds target multiple p53 mutants, one of the more interesting approaches from recent years is to develop "mutant-specific" drugs. PhiKan083 is a small molecule that binds to a small cleft in the C-terminus of p53-Y220C stabilizing the conformation of the core domain and restoring transactivation of p53 target genes (Boeckler et al., 2008). While it will take time to determine the utility of PhiKan083 as a clinical agent, it is a beautiful proof-of-principle of rational drug discovery based on the crystal structure of a mutant p53 and, although it must be noted that this cleft is probably unique to Y220C, a similar approach to design drugs specific to hotspot mutants of p53 may be a promising avenue in the future.

Wild-type p53 has now secured its place as a critical player in cancer biology, but before we move on to the role of mutant p53 in tumors, it is important to review how exactly p53 acts as a tumor suppressor.

Function of wild-type p53

Wild-type p53 can be activated by a number of cellular stressors including DNA damage, hypoxia and oncogene activation (Vousden and Lu, 2002). Following activation, wild-type p53 normally functions as a sequence-specific transcription factor to inhibit cell cycle progression, promote senescence or induce apoptotic cell death (Prives and Hall, 1999; Vousden and Lu, 2002; Vousden and Prives, 2009).

The p53 protein possesses an acidic N-terminal transactivation domain (now recognized to be two distinct transactivation subdomains), a proline-rich domain, a centrally located sequence

specific DNA binding domain, followed by an oligomerization domain and a basic C-terminal regulatory domain (Figure 1.1) (Laptenko and Prives, 2006). Wild-type p53 functions as a homotetramer in cells, binding to p53 response elements composed of two decamers separated by a spacer of 0-14 nucleotides (5'-RRRCWWGYYYn_{0-14}RRRCWWGYYY-3')[1] (el-Deiry et al., 1992; Funk et al., 1992; Riley et al., 2008). Wild-type p53 has been shown to transactivate myriad genes, the products of which mediate the downstream cellular outcomes of p53 activation such as cell cycle arrest (*CDKN1A*, *MIR34A*, etc.), senescence (*CDKN1A*, *PAI1*, etc.) and apoptosis (*PUMA*, *BAX*, etc.) (Prives and Hall, 1999; Riley et al., 2008; Vousden and Lu, 2002).

Each of these cellular outcomes has been shown to be important for the tumor suppressive ability of wild-type p53. There is substantial evidence to support a role for apoptosis, or programmed cell death, in the tumor suppressive function of p53. For example, mice in which the proline-rich domain of p53 had been deleted (this mutant protein lacks the ability to induce cell-cycle arrest, but retains the ability to induce programmed cell death) were still efficiently protected from spontaneous tumor development, suggesting that the ability to induce apoptosis is critical to the tumor suppressive ability of p53 (Toledo et al., 2006). However, a second mouse model argues that cell-cycle arrest plays at least a partial role in tumor suppressive function. A rare tumor-derived mutant of p53 (p53-R175P)[2] was identified that was able to induce cell-cycle arrest, but was unable to induce apoptosis (Rowan et al., 1996). Mice expressing the murine

[1] A=adenine, T=thymine, C=cytosine, G=guanine, R=purine, Y=pyrimidine, W=A/T, n=any nucleotide.

[2] This nomenclature will be used throughout this text. The first letter represents the amino acid present in the wild-type protein, the number represents the amino acid position number counting from the N-terminus and the last letter represents the amino acid present in the mutated protein. In this case, R175P designates an Arginine mutated to a Proline at position 175 in the p53 protein.

equivalent to this p53 mutant (p53-R172P) demonstrate a delay in spontaneous tumor formation, suggesting that cell-cycle arrest and chromosome stability also protect against tumor development (Liu et al., 2004a). These and many other data support the notion that wild-type p53 is unequivocally a tumor suppressor. However, biology, it seems, is never satisfied with a simple answer.

Gain-of-Function Hypothesis

One of the more interesting aspects within the p53 field emerged to help explain many of the seemingly contradictory results from the first decade of p53 research, when p53 was widely considered to be an oncogene. Unlike most tumor suppressor genes, which are predominantly inactivated as a result of deletion or truncation (Weinberg, 1991), the vast majority of cancer-associated mutations in *TP53* are missense mutations, single base-pair substitutions that result in the translation of a different amino acid in that position in the context of the full-length protein. Interestingly, these missense mutations tend to cluster at a number of "hot-spot" residues in the DNA binding domain (Figure 1.1) (Cho et al., 1994; Harris and Hollstein, 1993; Petitjean et al., 2007a). This is in striking contrast to the majority of tumor suppressors (examples include *RB1*, *APC*, *NF1*, *NF2* and *VHL*), the primary mutations in which are deletion or nonsense, leading to little/no expression of the respective proteins (Levine et al., 1995). While wild-type p53, under unstressed conditions, is a very short-lived protein, these missense mutations lead to the production of full-length altered p53 protein with a prolonged half-life (Strano et al., 2007). Many of these stable mutant forms of p53 exert a dominant-negative effect on the remaining wild-type allele, serving to abrogate the ability of wild-type p53 to inhibit cellular transformation, particularly when the mutant protein is expressed in excess of its wild-type counterpart (Brosh and Rotter, 2009; Oren and Rotter, 2010). Importantly, missense mutations

in p53 are usually followed by loss of heterozygosity (LOH) at the corresponding locus, suggesting that there is a selective advantage conferred by losing the remaining wild-type p53, even after one allele has been mutated (Baker et al., 1990; Brosh and Rotter, 2009). These observations, among others, led to the "gain-of-function" hypothesis, which states that mutation of *TP53* is not equivalent to simply losing wild-type p53 function. Instead, the strong selection for maintained expression of a select group of mutant p53 proteins suggests a positive role for certain p53 mutants in tumorigenesis.

While inactivating missense mutations in p53 may be selected for during tumor progression due to their ability to act as dominant-negative inhibitors of wild-type p53, there is also clear evidence that mutant p53 can exert oncogenic, or gain-of-function, activity independent of its effects on wild-type p53 (Sigal and Rotter, 2000). For example, when tumor-derived mutants of p53 are expressed in non-transformed, otherwise p53-null, cells, this confers the ability to form tumors in nude mice, whereas the parental cells cannot (Dittmer et al., 1993; Wolf et al., 1984). As was the case in confirming the tumor-suppressive role of wild-type p53, mouse models were essential in demonstrating that mutation of p53 is not equivalent to simply losing wild-type p53 function. Almost a decade after the original p53 knockout mice were generated, two groups independently engineered "knock-in" mouse models of Li-Fraumeni syndrome. These groups employed mice expressing tumor-derived mutants of p53, in which the murine equivalents of two of the most frequent "hot-spot" mutations in p53 were inserted into the endogenous *Trp53* locus using homologous recombination, in an attempt to better recapitulate the human disease. Remarkably, both knock-in mutations led to an altered tumor spectrum, in addition to more metastatic tumors (Lang et al., 2004; Liu et al., 2000; Olive et al., 2004). Similarly, in an analysis of Li-Fraumeni syndrome patients, germline missense mutations

in *TP53* have been shown to be associated with an earlier age of onset (approximately nine years), when compared with germline deletions in *TP53*, suggesting a gain-of-function effect of missense p53 mutants in human tumors (Bougeard et al., 2008).

More recently, mutations in p53 have been implicated in diverse aspects of tumorigenesis including proliferation, survival, chemoresistance, limitless replication, somatic cell reprogramming, inflammation, migration, invasion, angiogenesis and metastasis (Adorno et al., 2009; Bossi et al., 2006; Brosh and Rotter, 2009; Di Agostino et al., 2006; Irwin et al., 2003; Mizuno et al., 2010; Muller et al., 2009; Oren and Rotter, 2010; Sampath et al., 2001; Sarig et al., 2010; Stambolsky et al., 2010; Weisz et al., 2007a). In addition, multiple studies have also demonstrated that the mutational status of p53 is a strong predictor for poor outcomes in many types of human tumors, particularly breast cancer (Elledge et al., 1993; Langerod et al., 2007; Olivier et al., 2006; Petitjean et al., 2007a).

As noted by Oren and Rotter, while the concept of mutant p53 gain-of-function is now well established, the exact criteria for what constitutes "gain-of-function" can still be quite confusing (Oren and Rotter, 2010). The current knowledge on mutant p53 gain-of-function comes largely from experiments in which researchers overexpressed mutant versions of p53 in cell culture or mouse tumor models. As discussed above, many cancer-associated p53 mutants can exert dominant-negative effects on wild-type p53, so any "gain-of-function" experiments performed in the presence of wild-type p53 must be interpreted with extreme caution. To thoroughly demonstrate gain-of-function, two main approaches are now utilized: (1) expression of mutant forms of p53 in an otherwise p53-null background or (2) RNA interference (RNAi) to deplete the levels of an endogenous mutant p53 from cancer cells that have lost the wild-type p53 allele. This is an important point to make, as any experiments performed in cells that harbor

wild-type p53 do not necessarily prove a gain-of-function, but may simply reflect a dominant-negative effect.

Pro-Oncogenic Roles of Mutant p53

Recent years have seen an explosion of proposed roles for p53 mutations in tumorigenesis and diverse mechanisms to explain these roles for mutant p53 proteins. It is important to keep in mind that while the term "gain-of-function" has grown in popularity to explain observed effects of mutant p53 proteins, this may not necessarily be the best terminology, as will be seen below. There are three primary categories of proposed mechanisms to explain the pro-oncogenic effects of mutant p53 in tumor biology, which are not mutually exclusive (Brosh and Rotter, 2009). First, tumors may select for mutation of p53 solely for loss of wild-type p53 tumor suppressive activity. Second, mutants of p53 may lose certain tumor suppressive functions of wild-type p53, while retaining and/or exaggerating other aspects of normal wild-type p53 function. Finally, these mutant p53 proteins may acquire truly neomorphic, or gain-of-function, activities which actively promote tumor growth.

Gain-of-function activities of mutant p53 are now well-established and these are commonly attributed to one of two primary mechanisms: (1) an interaction between mutant p53 and cellular proteins or (2) mutant p53-mediated regulation of novel target genes. Of course, these two options are not mutually exclusive; for example, tumor-derived mutants of p53 have been shown to interact with a number of cellular partners, including cellular transcription factors (such as: NF-Y, Sp1, VDR, NF-κB and Ets-1) and affect their transcriptional activity. This will be discussed in greater detail in a later section.

Mutant p53 interacting partners

While wild-type p53 acts predominantly as a transcription factor, a number of the described gain-of-function activities of mutant p53 are mediated through non-transcriptional processes (Brosh and Rotter, 2009). Mutant p53 can form aberrant protein complexes with a number of interacting partners, perturbing their activity. A few of the more prominent examples will be reviewed below.

MRE11 was recently identified as a novel interacting partner with two hot-spot p53 mutants (p53-R248W and p53-R273H) which had been "knocked-in" to a humanized p53 allele in mice ($p53^{hki}$) (Song et al., 2007). The humanized ("Hupki") p53 mouse model encodes a human/mouse chimeric p53 protein (amino acids 33-332 are human, while the N- and C-termini are composed of murine p53) (Luo et al., 2001). The interaction between the nuclease MRE11 and mutant p53 was demonstrated to confer a gain-of-function by promoting genetic instability in tumors and pre-tumorigenic lesions in the knock-in mice. This interaction impaired the recruitment of the Mre11-Rad50-NBS1 (MRN) complex to double-strand breaks and diminished the activation of ataxia-telangiectasia mutated (ATM), enabling the persistence of unrepaired DNA breaks (Song et al., 2007).

Two recently described mutant p53 interacting partners enhance the oncogenic activity of tumor-derived p53 mutants. The promyelocytic leukemia (PML) protein is the major component of nuclear PML bodies, which have been implicated in proliferation and programmed cell death. Mutant p53 physically associates with PML and this interaction facilitates the transcriptional activity of mutant p53 (Haupt et al., 2009). In addition, mutant p53 interacts with the prolyl isomerase Pin1, which binds to phosphorylated serine/threonine residues next to a proline,

isomerizes the intervening peptide bond, resulting in conformational changes that affect protein stability and activity. Pin1 was demonstrated to cooperate with mutant p53 to promote a "pro-aggressiveness" transcriptional program that increased the migration and invasion of breast cancer cells (Girardini et al., 2011). Furthermore, this interaction was shown to be required for certain mutant p53 gain-of-function activities since Pin1 was necessary for oncogenic transformation of primary mouse fibroblasts by mutant p53 in cooperation with oncogenic H-Ras^{G12V} and deletion of Pin1 attenuated the mutant p53 gain-of-function phenotype in mice (Girardini et al., 2011).

While the mutant p53 interactome is rapidly expanding, the most widely studied mutant p53 interacting partners remain the p53 family members, p63 and p73.

The p53 family: p63 and p73

The p53 family consists of three proteins: p53, p63 and p73, which are homologous in the three primary domains of p53: transactivation domain, DNA-binding domain and C-terminal oligomerization domain (Li and Prives, 2007). The highest degree of homology exists in the DNA-binding domains; p63 and p73 exhibit 60% and 63% homology with the DNA-binding domain of p53, respectively (Irwin and Kaelin, 2001). The N-terminal transactivation domain is only 30% identical between p53 and p73 and 22% identical between p53 and p63, while the oligomerization domains of both p63 and p73 exhibit 38% identity with that of p53 (Irwin and Kaelin, 2001). To complicate matters, both p63 and p73 are expressed as multiple isoforms, either through alternate promoter usage (N-terminal TA or ΔN forms for both p63 and p73) or through alternative splicing of the C-termini (p63-α, -β, -γ and p73-α, -β, -γ, -δ, -ε, -ζ) (Pietsch et al., 2008).

As mentioned previously, wild-type p53 functions as a tetramer, so it came as no surprise that p63 and p73 were shown to form homo- and hetero-tetramers with each other, although neither p63 nor p73 could interact with wild-type p53 (Davison et al., 1999). However, shortly thereafter a number of tumor-derived mutants of p53 were demonstrated to interact with both p63 and p73 (Di Como et al., 1999; Gaiddon et al., 2001; Irwin et al., 2003; Marin et al., 2000; Strano et al., 2002; Strano et al., 2001) and this interaction has been invoked to explain many of the gain-of-function effects of mutant p53. For example, binding to p63 and/or p73 has been linked to the ability of certain tumor-derived mutants of p53 to promote chemoresistance, migration, invasion and metastasis (Adorno et al., 2009; Muller et al., 2009; Sampath et al., 2001). As mentioned above, the altered tumorigenic phenotypes observed in mutant p53 knock-in mice ($p53^{R172H/-}$ and $p53^{R270H/-}$, compared to $p53^{-/-}$) provide some of the most compelling evidence for a mutant p53 gain-of-function in tumorigenesis (Olive et al., 2004). Interestingly, both $p53^{+/-}/p63^{+/-}$ and $p53^{+/-}/p73^{+/-}$ double mutant mice display an altered tumor spectrum and display a more metastatic phenotype, highly reminiscent of the phenotype observed in the mutant p53 knock-in models (Flores et al., 2005). This is in line with the idea that gain-of-function p53 mutants act at least in part by inhibiting the function of p63/p73.

Interestingly, the site of interaction between mutant p53 and p63/p73 is likely not to occur through the oligomerization domains, as was initially expected. Instead, early reports demonstrated that this interaction was mediated through the DNA-binding domains, as the core domain of mutant p53 was sufficient to interact with p63 or p73 in co-immunoprecipitation assays (Gaiddon et al., 2001; Strano et al., 2002; Strano et al., 2001). These observations provide a potential explanation for the specificity of the interaction between p63/p73 and tumor-derived mutants of p53 and not wild-type p53. The vast majority of missense mutations in p53

occur within the core DNA-binding domain and many of the hot-spot mutants of p53 have been shown to produce either local or global conformational changes (Wong et al., 1999). However, it should be noted that p63/p73 also interact with "DNA-contact" mutants of p53 (albeit less effectively than with "conformational" mutants), which are reported not to dramatically alter the structure of the mutant p53 protein (Li and Prives, 2007).

There are multiple lines of evidence that agree with this site of interaction, although the debate is not yet settled. In agreement with this explanation is the fact that there was a strong correlation between the observed ability of a tumor-derived mutant to bind to p63/p73 and the ability of that p53 mutant to be recognized by PAb240, which recognizes a cryptic epitope within the core domain of p53 (Li and Prives, 2007). In addition, wild-type p53, when denatured, was demonstrated to acquire the ability to interact with p73 (Bensaad et al., 2003). Finally, two peptides derived from the p73 DNA-binding domain were demonstrated to disrupt the interaction between p73 and tumor-derived mutants of p53 (Di Agostino et al., 2008). However, there have been several recent reports that have implicated the C-terminus as the primary determinant for the interaction between mutant p53 and p63/p73, at least in the case of certain tumor-derived mutants (Muller et al., 2009; Xu et al., 2011). Interestingly, several groups have demonstrated that a single nucleotide polymorphism (SNP) at codon 72 in p53 (this residue can be either arginine or proline) alters the ability of mutant p53 to interact with p73, as mutant p53 with an arginine at this residue has a much higher affinity for p73 than a mutant p53 with a proline (Marin et al., 2000).

As the interaction between mutant p53 and p63/p73 has been implicated in many pro-oncogenic effects of mutant p53, one of the more intriguing therapeutic approaches may be to disrupt this interaction, thus allowing p63 and/or p73 to function. RETRA is a small molecule

reported to do just that. In a screen hoping to identify small molecules that could activate transcription from a wild-type p53 reporter construct, the top compound, RETRA (RE-activation of Transcriptional Reporter Activity), was actually found to disrupt the mutant p53:p73 complex, allowing p73 to activate transcription from this reporter construct. This was elegantly demonstrated as RETRA-mediated activity was completely impaired when the cells bearing mutant p53 were depleted of p73 using RNAi and then treated with RETRA. This compound could also activate transcription of p53-target genes in mutant p53-bearing tumors *in vivo* and prevent xenografted tumor cell growth (Kravchenko et al., 2008). While this compound may not "re-activate" wild-type p53, it is tempting to speculate that perhaps inhibiting some of the gain-of-function effects of mutant p53 might be sufficient as a therapeutic approach for mutant p53 tumors.

Since p63 and p73 are both sequence-specific transcription factors, mutant p53 binding may alter the activity of these family members at their target gene promoters. Mutant p53 has traditionally been assumed to play an inhibitory role towards its family members, particularly when assayed using reporter constructs with a consensus p53 response element (Gaiddon et al., 2001). However, as more and more p63 and/or p73 target genes are uncovered, many of which are independent of wild-type p53, it remains to be seen if the relationship between mutant p53 and p63/p73 is always antagonistic.

Mutant p53 and transcription

Wild-type p53 is a potent transcriptional activator and the transactivation domain (TAD) of p53 can be subdivided into two subdomains, TAD1 contained within residues 1-40 and TAD2 contained within residues 41-61 (Chang et al., 1995; Walker and Levine, 1996). In fact, it has

been shown that wild-type p53 is dependent upon four critical hydrophobic amino acids in its amino terminus for transactivation activity, Leucine-22, Tryptophan-23, Tryptophan-53, Phenylalanine-54. When these four residues are mutated to polar amino acids (L22Q/W23S/W53Q/F54S) p53 transactivation capability is completely abolished (Candau et al., 1997; Lin et al., 1994; Venot et al., 1999; Zhu et al., 1998). The transactivation subdomains have been shown to be essential for binding to co-activators of p53 such as p300 (Teufel et al., 2007) and TATA-binding protein (Chang et al., 1995), in addition to mediating other regulatory interactions such as Mdm2 (Kussie et al., 1996; Lin et al., 1994) and Mdm4 (Shvarts et al., 1996).

Just as wild-type p53 primarily functions as a transcription factor, mutant p53 proteins have been shown to transactivate myriad genes involved in many different aspects of tumorigenesis (Brosh and Rotter, 2009; Strano et al., 2007; Weisz et al., 2007b). In addition, a number of studies have implicated the transactivation domains of p53 as critical for pro-oncogenic functions of p53 mutants (Frazier et al., 1998; Lin et al., 1995; Matas et al., 2001; Yan and Chen, 2010). While the transactivation targets of wild-type p53 tend to either promote cell cycle arrest/senescence or programmed cell death, the targets of tumor-derived p53 mutants are quite varied in terms of their biological effects. Mutant p53 activates a number of target genes that serve to increase proliferation, inhibit apoptosis, promote chemoresistance and impinge on key metabolic or cell-cell/cell-ECM signaling pathways (Table 1.1).

Tumor-derived p53 mutants have been demonstrated to transactivate *MYC* (Frazier et al., 1998), *CXCL1* (Yan and Chen, 2009), *PCNA* (Deb et al., 1992), *MAP2K3* (Bossi et al., 2008), *CCNA*, *CCNB*, *CDK1*, *CDC25C* (Di Agostino et al., 2008), *ASNS* (Scian et al., 2004), *E2F5*, *MCM6* (Scian et al., 2005), *IGF1R* (Werner et al., 1996), *STMN1* (Singer et al., 2007) and *EGFR*

(Ludes-Meyers et al., 1996), all of which can promote proliferation of cancer cells. Additionally, mutant p53 proteins can upregulate genes, the protein products of which serve to inhibit apoptosis or promote chemoresistance. For example, p53 mutants can transactivate *EGR1* (Weisz et al., 2004), *ABCB1* (also known as MDR1 or P-glycoprotein) (Chin et al., 1992; Lin et al., 1995; Sampath et al., 2001; Strauss and Haas, 1995), *IGF2* (Lee et al., 2000), *DUT* (Pugacheva et al., 2002), *BCL2L1* (also known as Bcl-xL) (Bossi et al., 2008), *TIMM50* (Sankala et al., 2011), *LGALS3* (Lavra et al., 2009) and *NFKB2* (Scian et al., 2005), which can all inhibit cell death. Finally, p53 mutants can upregulate genes with a variety of other cellular effects such as limitless replication (*TERT*) (Scian et al., 2004), matrix degradation (*MMP3 and MMP13*) (Buganim et al., 2010; Sun et al., 2000) and Rho GTPase signaling (*ARHGDIA*) (Bossi et al., 2008).

To date there is no unifying hypothesis to explain the ability of mutant p53 proteins to regulate such a wide variety of target genes, due in part to the absence of a defined "mutant p53 response element." However, there have been a number of proposed mechanisms that account for individual genes or classes of genes regulated by p53 mutants. One of the more common mechanisms seems to be that mutant forms of p53 can interact with other sequence-specific transcription factors, be recruited to the cognate binding site of that cellular transcription factor and either strengthen or dampen the target response. To this end, at least three mutants of p53 have been demonstrated to interact with the CCAAT-binding factor NF-Y, and this complex serves to upregulate NF-Y target genes such as *CCNA*, *CCNB*, *CDK1* and *CDC25C* (encoding cyclins and cyclin dependent kinases that serve to promote cell cycle progression) following adriamycin (also known as doxorubicin) treatment (Di Agostino et al., 2006). Recently, it was shown that the interaction between mutant p53 and NF-Y is mediated by TopBP1 (Liu et al.,

2011) and, as discussed above, the transcriptional activity of mutant p53 toward NF-Y target genes is enhanced by mutant p53's interaction with PML (Haupt et al., 2009). The ability of mutant p53 to upregulate *ABCB1* (also known as MDR1), a transmembrane protein characterized by an ATP-binding cassette which can efflux a variety of structurally and functionally distinct substrates and thus promote tumor chemoresistance (Bush and Li, 2002), has been shown to be dependent on a functional interaction with Ets-1 (Sampath et al., 2001). Mutant p53 can also interact with Sp1 bound to the consensus Sp1 response elements in the HIV-LTR and augment its activity (Chicas et al., 2000). Additionally, NF-κB target genes have been found to be significantly overrepresented in a ChIP-on-chip analysis of potential mutant p53 binding sites and a tumor-derived p53 mutant can enhance the transcriptional activity of NF-κB in response to TNF-α (Dell'Orso et al., 2011; Weisz et al., 2007a). Recently mutant p53 has also been shown to bind to the vitamin D receptor (VDR) and serve to synergistically upregulate vitamin D responsive genes such as *IGFBP3* and *CYP24A1* (Stambolsky et al., 2010).

Interestingly, wild-type p53 has been shown to interact with many of the same transcription factors: NF-Y, Sp1 and VDR (Bargonetti et al., 1997; Imbriano et al., 2005; Stambolsky et al., 2010). Likewise, wild-type p53 regulates many of the same target genes as described for mutant p53. However, the outcome of wild-type p53 binding is often exactly reciprocal to that mediated by mutant p53. Whereas mutant p53 augments the expression of NF-Y, Sp1, NF-κB, Ets-1 and VDR target genes, wild-type p53 most commonly represses these same target genes (Di Agostino et al., 2006; Lane and Levine, 2010; Peart and Prives, 2006; Sampath et al., 2001; Stambolsky et al., 2010; Sun et al., 2000; Werner et al., 1996). In partial explanation of this, Di Agostino et al. have shown that in response to adriamycin wild-type p53 and mutant p53 recruit different co-factors, the histone deacetylase HDAC1 in the case of wild-

type p53 and the histone acetyltransferase p300 in the case of mutant p53 (Di Agostino et al., 2006). This switch in epigenetic modifiers serves to increase histone acetylation and thus results in increased transcription in the presence of mutant p53 proteins.

While this mechanism, of co-opting cellular transcription factors to promote the transactivation of their target genes, provides some explanation for the activity of mutant p53, a critical question still remains: in cells where mutant p53 can be shown to interact with a particular transcription factor (e.g. NF-Y, Sp1, Ets-1, etc.), why are only a subset of their target genes affected by mutant p53? One can speculate that perhaps this is due to a necessary co-factor, in addition to mutant p53 and its partner transcription factor, and the complex is only present on certain promoters at any given time. Alternatively, perhaps the chromatin landscapes of certain promoters are particularly amenable to mutant p53 binding.

While a subset of mutant p53 target gene regulation can be explained by association with sequence-specific transcription factors, the different promoters activated by p53 mutants share little to no sequence homology. This has led to the hypothesis that mutant p53 recruitment may be determined by DNA structural motifs as opposed to recognition of a sequence-specific mutant p53 response element (Kim and Deppert, 2004, 2007). It has been shown that a number of tumor-derived mutants of p53 have a high affinity for nuclear matrix attachments regions (MARs), DNA sequences that bind with high affinity to the nuclear matrix, a salt and detergent-insoluble proteinacious structure (Koga and Deppert, 2000; Muller et al., 1996; Will et al., 1998). Matrix attachment regions are highly AT rich regions which promote structural alterations within chromatin and often adopt non-B DNA conformations (Kim and Deppert, 2004). In support of this hypothesis, mutant p53 proteins have been shown to bind to non-B DNA *in vitro* and *in vivo* (Brazdova et al., 2009; Gohler et al., 2005). Mutant p53 binding to DNA structure-specific

motifs is presumably a remnant of a wild-type p53 function, as it has now been shown that, in addition to sequence-specificity, wild-type p53 binds to DNA via multiple sequence non-specific interactions (Kim and Deppert, 2003). For example, wild-type p53 exhibits high affinity binding to double-stranded DNA, single-stranded DNA, secondary DNA structures and mismatched bases and DNA bulges (Kim and Deppert, 2003). Interestingly, the structure-selectivity of both wild-type and mutant p53 requires its C-terminus, which is rarely mutated in human tumors (Petitjean et al., 2007a; Petitjean et al., 2007b).

The findings that mutant p53 proteins can interact with target gene promoters through other transcription factors and in a DNA-structure-specific manner have been used to justify the absence of a well-described consensus mutant p53 sequence-specific response element. However, these attributes may in fact mask the existence of a mutant p53 response element if one exists. In other words, using genome-wide analyses to identify mutant p53 binding sequences, one could not distinguish between sequences that are bound through other transcription factors, through DNA-structural motifs or directly through a (hypothetical) mutant p53 response element.

What can we learn from mutational spectra of TP53?

Mutagenic events in DNA are generally accepted to arise from either external events (environmental mutagens) or internal events (resulting from replication errors, depurination, errors in repair, etc.) (Caron de Fromentel and Soussi, 1992). The nature of mutations arising from external (exogenous) events is determined primarily by the mutagenic agent. For example, ultraviolet (UV) light commonly induces pyrimidine-dimers in adjacent nucleotides, a common mutational event in skin carcinogenesis (Basset-Seguin et al., 1994). On the other hand, internal

(endogenous) mutations arise from spontaneous events and preferentially occur at CpG dinucleotides. This is likely due to the fact that the cytosine in this dinucleotide is frequently methylated and spontaneous deamination can occur changing the cytosine to uracil and the resulting U:G mispair can give rise to a C:G to T:A transition (Barnes and Lindahl, 2004).

As mentioned previously, there are certain "hot-spot" missense mutations in the *TP53* gene, residues which are mutated at a much higher frequency than expected by chance. Together these account for nearly one third of all p53 mutations (Brosh and Rotter, 2009). This fact is often cited to support a gain-of-function hypothesis as it is suggestive of a selective advantage of having certain mutations. The amino acids generally regarded as hot-spots (R175, G245, R248, R249, R273, R282) are found mutated in many malignancies. Endogenous mutations frequently arise from spontaneous deamination of methylated CpG dinucleotides and interestingly, the codons at four of these hot-spot residues contain CpG dinucleotides. A transition (C to T or G to A) can occur following deamination of the 5-methylcytosine and not surprisingly, this is exactly the type of nucleotide change observed at these hot-spots in p53 (Caron de Fromentel and Soussi, 1992). However, the p53 gene contains many other CpG dinucleotides that are rarely found mutated in cancer, supporting the notion that these hot-spots are functionally important to either inactivate wild-type p53 or that these substitutions offer distinct activities to the neomorphic protein.

However, other p53 mutations occur only in specific tumor types, suggesting that environmental mutagens leave their mark on p53 in a tumor and tissue-selective manner. For example, lung, liver and skin cancer all bear unique mutation distributions in *TP53*, which may be a result of carcinogens. Hepatocellular carcinoma in certain developing nations is marked by a much higher rate of p53-R249S mutations than other tumor types or even liver cancers in

developed nations (Staib et al., 2003). In addition to the fact that this mutation is deleterious to p53 function, it has a strong association with exposure to Aflatoxin B1. Aflatoxins are produced by fungal species (*Aspergillus flavus* in the case of Aflatoxin B1), and commonly contaminate food supplies in developing areas of sub-Saharan Africa and Asia. In support of this association playing a causal role in hepatocellular carcinogenesis, Aflatoxin B1 has been shown to induce the same R249S mutation in *TP53* when cultured with hepatocytes *in vitro* (Staib et al., 2003). Similarly, a strong association has been demonstrated between benzo(a)pyrene exposure (found in cigarette smoke) and G:C to T:A transversions. These often occur in lung cancer at amino acid positions in p53 that are not otherwise frequently mutated (i.e. 157 and 158) (Toyooka et al., 2003). In fact, it has been proposed the mutational spectra for different tumor types might represent a "fingerprint" for the mutational agent responsible for tumorigenesis (Lasky and Silbergeld, 1996).

Mutant p53: One name, many proteins

While many groups choose to use the generic term "mutant p53" to designate any tumor-derived p53 mutant, it is important to recognize that not all p53 mutants are equal. Some mutants of p53 will exert gain-of-function effects, but many other p53 mutants will be selected for during tumorigenesis simply because they abolish wild-type p53 activity. p53 mutations can generally be classified as either "conformational" or "DNA-contact" mutants (Brosh and Rotter, 2009). DNA-contact (or class I) mutants, exemplified by p53-R273H, are missense mutations in the amino acid residues that normally make direct contact with target DNA sequences. Conformational (or class II) mutants, typified by p53-R175H, are those missense mutations that disrupt the structure of the p53 protein on either a local or global level (Sigal and Rotter, 2000; Soussi and Lozano, 2005).

This distinction is potentially important, particularly when one attempts to make generalizations about mutant p53 proteins. Many target genes have been identified in the context of particular missense mutations in p53 (Table 1.1), but are now referred to as mutant p53 target genes. Similarly, many mutant p53 binding partners bind with a higher affinity to a subset of tumor-derived p53 mutants. For example, conformation mutants of p53 tend to have a higher affinity for p63/p73, as well as hsp70, and can be readily identified by the conformation-specific PAb240 (Gaiddon et al., 2001; Hinds et al., 1990). In fact, even different amino acid substitutions at the same position in the p53 protein can have dramatically different phenotypic effects. For example, p53-R248Q, but not p53-R248W (both of which are common tumor-derived mutants), was able to confer invasive ability when overexpressed in p53-null cells (Yoshikawa et al., 2010). Additionally, as already mentioned, p53-R175H and p53-R175P have dramatically different properties, which is highlighted by the different phenotypes they produce when "knocked-in" to the endogenous locus in mice (Donehower and Lozano, 2009). Particularly in the case of attempting to identify a "mutant p53 response element" (if one can be identified), it is likely that each contact mutant of p53 may have slightly different sequence-specificity since these mutations affect the amino acid residues that directly contact the DNA backbone (Brosh and Rotter, 2009).

Stabilization of Mutant p53 in Tumors

Mutant p53 proteins are often found at extremely high levels in tumors. In fact, positive immunohistochemical staining of p53 in tissue sections is commonly used as a surrogate for detecting a missense mutation in *TP53* (although this is an imperfect marker) (Alsner et al., 2008; Bartek et al., 1990). Wild-type p53 is maintained at very low levels in most cells due primarily to tight regulation by the Mdm2 E3 ubiquitin ligase, itself a p53 target gene thus

creating a negative feedback loop (Vousden and Prives, 2009). Mutant forms of p53, on the other hand, are quite stable and often accumulate in tumor cells. Until recently, the prevailing hypothesis to explain the observation of high levels of mutant p53 in tumors was that p53 mutation broke this feedback loop by rendering the p53 mutant protein incapable of transactivating *MDM2* (Oren and Rotter, 2010). Arguing against this hypothesis is the fact that in the mutant p53 knock-in mouse models, although all tissues contained the mutant allele, these mice did not accumulate mutant p53 in most normal tissues (Lang et al., 2004; Olive et al., 2004). On the other hand, mutant p53 was observed at high levels in a subset of the tumors in these same mouse models (Terzian et al., 2008). Interestingly, when the mutant p53 knock-in mice were crossed into an Mdm2-null background, the mutant p53 protein was stabilized in some normal tissues, albeit not all (Terzian et al., 2008). Together, these findings strongly suggest that levels of mutant p53 can be regulated by similar mechanisms as that of the wild-type p53 protein, but additional events occur during tumorigenesis that are necessary to abrogate this regulation and lead to accumulation of mutant p53.

In line with this idea, at least one report has demonstrated an enhanced mutant p53 gain-of-function phenotype following common chemotherapeutic agents (Di Agostino et al., 2006). Similarly, it was recently reported that in a large randomized clinical trial to investigate the effects of cisplatin-based adjuvant chemotherapy in non-small cell lung carcinoma (NSCLC) patients following tumor resection, *TP53* mutational status had a significant predictive impact for response to therapy. While *TP53* status had no prognostic significance in the absence of adjuvant chemotherapy, patients bearing a mutant p53 had a significantly reduced disease-free interval and overall survival only after undergoing treatment with cisplatin (Goldstein et al., 2011). These findings may have important clinical repercussions, not just in the setting of

traditional chemotherapeutics, but particularly as Mdm2 antagonists are under investigation as therapeutic agents (Prives and White, 2008).

As high levels of mutant p53 have been shown to be important for many aspects of tumorigenesis, one potential therapeutic approach is to target cellular proteins responsible for mutant p53 stabilization. One recent development has done just that, in targeting HSP90. It has long been observed that mutant forms of p53 interact with heat shock proteins in tumors (Sepehrnia et al., 1996) and that this interaction affects Mdm2-mediated turnover of mutant p53 (Peng et al., 2001). Recently, RNAi-mediated depletion or pharmacologic inhibition of HSP90 has been shown to destabilize mutant p53, counteracting many of its gain-of-function effects (Li et al., 2011a; Li et al., 2011b).

p53 and metabolism

Of the many differences between normal and tumorigenic cells, some of the earliest recognized were metabolic changes that accompanied transformation (Vousden and Ryan, 2009). The most famous of which was first described by Otto Warburg; specifically that transformed cells rely almost solely on anaerobic glycolysis as opposed to oxidative phosphorylation to obtain energy, a phenomenon which has come to be known as the Warburg effect (Warburg, 1956; Warburg et al., 1927). However, it should be noted that although the Warburg effect has dominated the focus of cancer metabolism over the past decades, deregulation of sterol biosynthesis has also been observed in a high proportion of cancer cells and has been put forth as hallmark of transformation (Siperstein, 1970). Recent years have seen a resurgence of interest in metabolic changes associated with cancer as many of these have been shown to play a critical

role in maintaining the malignant state of tumors and the p53 protein is emerging as a key regulator of cellular metabolism (Vousden and Ryan, 2009).

Wild-type p53 has been shown to impinge upon glucose metabolism, fatty acid oxidation, glutaminolysis and antioxidant response, so it is perhaps no surprise that p53 mutant tumors exhibit altered metabolism (Maddocks and Vousden, 2011). p53 can directly inhibit the expression of two glucose transporters, GLUT1 and GLUT4, as well as destabilize the phosphoglycerate mutase (PGM) protein (Maddocks and Vousden, 2011). In addition, p53 upregulates TIGAR (*TP53*-induced glycolysis and apoptosis regulator), which functions as a fructose-2,6-bisphosphatase, thereby fettering glycolytic flux (Vousden and Ryan, 2009). The action of p53 on this pathway not only limits glycolysis, but also promotes flux through the pentose phosphate pathway (PPP) (Maddocks and Vousden, 2011). Although glucose is the primary energy source for most cells, glutamate can also feed into bioenergetic pathways through the action of glutaminase. Recently, glutaminase 2 (encoded by *GLS2*) has been shown to be a wild-type p53 target gene (Hu et al., 2010; Suzuki et al., 2010). In addition, wild-type p53 promotes multiple players in oxidative phosphorylation, thus it plays a central role in determining the mode by which cells obtain energy (Vousden and Ryan, 2009).

TP53 and breast cancer

Breast cancer is the most common malignancy diagnosed among women and the second-most cause of cancer-related death among women in the United States (Jemal et al., 2009). At the current rates, one in eight women born today in the U.S. will develop invasive breast cancer at some point during her lifetime (Warner, 2011). Over the past twenty years, the mortality associated with breast cancer has decreased dramatically in a number of developed nations, most

likely due to earlier detection and better adjuvant therapies; however, as of 2009 one in five diagnosed with invasive breast cancer will still die of the disease (Jemal et al., 2009; Warner, 2011).

TP53 mutation is a common and early event in breast carcinogenesis. It is found to be altered in 25-40% of breast cancer cases and is often accompanied by subsequent loss of the wild-type allele (Borresen-Dale, 2003). Individuals with Li-Fraumeni Syndrome, who inherit germline mutations in *TP53*, are predisposed to many types of cancers; however, the most frequently diagnosed is premenopausal breast cancer (Varley et al., 1997). Consistent with the hypothesis that p53 mutation is an early step in mammary tumorigenesis, multiple studies have found that p53 is mutated in up to 30-40% of cases of high-grade ductal carcinoma *in situ* (DCIS), a pre-invasive breast lesion (Done et al., 2001; Ho et al., 2000; Zhou et al., 2009).

Breast cancer is thought to arise from mammary epithelial cells, found in structures referred to as acini, which collectively form terminal ductal lobular units (TDLU). Each acinus consists of a single layer of polarized luminal epithelial cells surrounding a hollow lumen (Figure 1.2) (Allred et al., 2001; Bissell et al., 2002). Breast carcinogenesis proceeds through a number of well described steps, from ductal carcinoma *in situ* (DCIS) to invasive breast cancer and mammary tissue architecture is invariably disrupted during breast tumor progression (Debnath et al., 2003a). As discussed above, recent reports have connected the mutant p53 gain-of-function hypothesis to breast cancer, demonstrating that mutant p53 can lead to increased invasion, migration and metastasis (Adorno et al., 2009; Muller et al., 2009); yet mutant p53 induced phenotypic alterations in mammary tissue architecture have not been fully explored.

Modeling the mammary gland: From 2D to 3D culture

The human mammary gland is composed of an extensive network of arborized ducts lined by epithelial cells, surrounding a hollow lumen. *In vivo,* this epithelium is actually comprised of an inner layer of luminal epithelial cells, which is immediately surrounded by an outer layer of myoepithelial cells. The bilayered structure of the mammary epithelium exhibits apicobasal polarization, with the luminal epithelial cells on the apical side (facing the lumen of the acinus) and the myoepithelial cells making up the basal layer, in close proximity to the basement membrane (Bissell et al., 2002). This mammary epithelium is embedded in a vast sea of adipose cells, interstitial dense connective tissue, loose connective tissue and endothelial cells, which collectively make up the mammary stroma (Hansen and Bissell, 2000).

While traditional cell culture has provided deep insight into the process of breast carcinogenesis, a fundamental limitation of this approach is that *in vitro* culture conditions create an environment for the cell that markedly differs from the microenvironment that they would experience *in vivo* (Vargo-Gogola and Rosen, 2007). It has long been recognized that when cells are maintained on glass or plastic tissue culture substrata, they rapidly lose many of their differentiated characteristics. For example, when primary mammary epithelial cells from a late-pregnant, lactating mouse are extracted and placed in traditional cell culture conditions, they no longer exhibit the prototypical signs of a secretory breast cell (extensive rough endoplasmic reticulum, well-developed Golgi apparatus and abundant casein synthesis), nor do they maintain their characteristic organization into three-dimensional glandular structures (Emerman et al., 1977). In the mid-1970s, it was demonstrated using floating collagen gels that, given the proper microenvironmental cues, it was possible to restore a number of differentiated functions to cells in culture (Bissell, 1981; Elsdale and Bard, 1972; Emerman et al., 1977; Hay and Dodson, 1973;

Lee et al., 1984; Michalopoulos and Pitot, 1975; Yang et al., 1980). Following these early observations, it was postulated that the functional unit in higher organisms should be thought of not simply as a single cell, but a cell in combination with its neighboring extracellular matrix (Bissell et al., 1982).

Pioneering work by the Bissell group established a system utilizing mammary epithelial cells grown in a laminin-rich extracellular matrix (ECM), in which they form structures highly reminiscent for many of the aspects of acinar structures found *in vivo* (Barcellos-Hoff et al., 1989; Bissell et al., 2005; Petersen et al., 1992; Shaw et al., 2004). As opposed to the early studies using floating collagen gels, subsequent studies have primarily utilized a three-dimensional extracellular matrix (3D ECM) system, in which mammary epithelial cells are embedded in a reconstituted laminin-rich basement membrane derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma (Barcellos-Hoff et al., 1989; Kleinman et al., 1986), although a number of other 3D culture systems are also employed. Commonly referred to as Matrigel, this laminin-rich ECM is composed primarily of laminin 1 (now referred to as laminin-111), collagen IV, heparan sulfate proteoglycan, nidogen and entactin (Kleinman et al., 1986). The laminin-rich ECM more faithfully recapitulates the microenvironment of the human mammary gland and allows normal mammary epithelial cells to develop into polarized glandular structures with hollow lumens, which can even be induced to synthesize and vectorially secrete milk proteins if given the correct lactogenic signals (Barcellos-Hoff et al., 1989). Therefore, although cells often loose tissue-specific functions in cell culture, if these cells are placed in a more physiologically relevant context, they can regain many features of their *in vivo* counterparts. Importantly, one of the hallmarks of tumorigenesis is disruption of normal tissue architecture and the dysregulation of many tissue-specific functions (Bissell, 2007; Debnath et

al., 2003a). In fact, pathologists have long recognized that certain histological patterns correlate strongly with patient prognosis (Hebner et al., 2008).

When grown as monolayer cultures, non-tumorigenic and tumorigenic cells are essentially indistinguishable. However, a multitude of studies have demonstrated that if these cells are cultured using a 3D laminin-rich ECM, one can readily distinguish normal and tumorigenic tissue by their morphological appearance (Kenny et al., 2007; Martin et al., 2008; Petersen et al., 1992; Wang et al., 1998; Weaver et al., 1997).

Modeling breast tumorigenesis in 3D culture

The importance of studying the effect of genetic alterations on tissue architecture in a microenvironment that faithfully recapitulates the *in vivo* microenvironment has become increasingly recognized. The 3D laminin-rich ECM model system has been used extensively to model the effects of oncogene expression (or depletion of tumor suppressors) on the three-dimensional morphogenesis of non-malignant mammary epithelial cells (Muthuswamy et al., 2001; Wrobel et al., 2004; Zhan et al., 2008).

In addition to primary mammary epithelial cells (MECs), a number of non-transformed, immortalized mammary epithelial cell lines can be induced to undergo acinar morphogenesis when plated in 3D culture. These include human S1 (from the HMT-3522 progression series) and MCF10A mammary epithelial cell lines, as well as murine Eph4 and SCp2 mammary epithelial cell lines (Muthuswamy et al., 2001; Niemann et al., 1998; Roskelley et al., 1994; Weaver et al., 1997). These non-malignant mammary epithelial cells undergo a well-characterized progression of three-dimensional morphogenesis (Debnath et al., 2003a).

Multiple phenotypic alterations associated with mammary tumorigenesis have been studied using the 3D culture system and many of the underlying molecular mechanisms governing these phenotypic effects have now been elucidated. We will explore a number of these phenotypic alterations in further detail below, including luminal filling, loss of apicobasal polarization, circumvention of growth arrest, disorganization and the invasive behavior of tumor cells. In addition, we will also examine the pathways/proteins that have been implicated as being necessary to maintain the malignant state of tumorigenic breast cells. With these in mind, two complementary approaches have been used to extensively study breast carcinogenesis in 3D culture, each of which will be discussed in the context of individual proteins below. The first is introduction of putative oncogenes or depletion of putative tumor suppressors to examine their effect on otherwise non-malignant mammary epithelial cells and the second is modulation of the activity and/or levels of pathways/proteins that can restore a more normal three-dimensional acinar-like morphology to disorganized breast cancer cells (referred to hereafter as a "phenotypic reversion").

Luminal clearance

A common feature of most glandular epithelial tumors is absence of a hollow lumen (Debnath and Brugge, 2005). In fact, one of the earliest stages of breast cancer is ductal carcinoma *in situ*, which is defined by an accumulation of cells within the luminal space. One of the primary mechanisms by which at least MCF10A cells undergo luminal clearance is through a specific form of apoptosis known as anoikis (Debnath et al., 2002). Anoikis is a unique form of programmed cell death which occurs in cells that detach from the basement membrane (Boudreau et al., 1995). Bim, a pro-apoptotic BH3-only Bcl family protein, has been implicated in anoikis and depletion of Bim from MCF10A cells inhibits both anoikis and delays luminal

clearance in 3D culture (Reginato et al., 2005; Reginato et al., 2003). However, lumen formation occurs *in vivo* even in the absence of Bim, demonstrating that there are alternative mechanisms of lumen formation (Mailleux et al., 2007). For example, recent work has demonstrated that reactive oxygen species (ROS) can lead to formation of a hollow lumen in the setting of apoptotic inhibition (Schafer et al., 2009). In fact, lumen formation has even been shown to spontaneously occur within small clusters of epithelial cells, suggesting that there may be intrinsic mechanisms to generate a hollow lumen (Hall et al., 1982; Ojakian et al., 1997).

Mechanistic insights into the process of lumen formation using MCF10A cells grown in 3D culture suggest that either an inhibition of apoptosis or an increase in cell proliferation alone is not sufficient to prevent luminal clearance; however, the combination of the two can prevent formation of a hollow lumen (Debnath et al., 2002). For example, forced expression of anti-apoptotic proteins in MCF10A cells (i.e. expression of Bcl-2 or Bcl-xL) does not prevent lumen formation, instead this only delays lumen formation by several days (Debnath et al., 2002). Similarly, expression of pro-proliferative factors such as Cyclin D1 or inactivation of critical cell cycle checkpoint proteins such as the retinoblastoma protein Rb (by the E7 viral product of human papilloma virus 16), increases proliferation in MCF10A 3D structures, but does not lead to luminal filling due to a compensatory increase in luminal apoptosis (Debnath et al., 2002). There have now been numerous proteins and/or pathways which have been implicated in disruption of acinar morphogenesis, a subset of which have been shown to be necessary to maintain the malignant state of breast cancer cells (Table 1.2). Of particular note to this study, luminal clearance in the mammary gland does not appear to be affected by p53 loss, suggesting that luminal apoptosis can occur though p53-independent means (Humphreys et al., 1996).

Driving Tumorigenesis: effects on mammary tissue architecture

As we examine the myriad putative oncogenes and tumor suppressors that have been studied using the 3D culture model, we will travel inward from the extracellular environment to cell surface proteins to cytoplasmic signaling molecules to primarily nuclear factors. While this organization is helpful in conceptualizing these factors, it is important to keep in mind one of the key principals garnered from the 3D culture system, dynamic reciprocity. By this, we mean that there is a constant flux of information transmitted from extracellular to intracellular environments as well as from intracellular to extracellular environments, thus one must always keep in mind the interplay between these compartments.

Extracellular Factors

The matrix metalloproteinase (MMP) family comprises a large number of secreted or membrane-associated proteins that cleave a wide range of extracellular matrix, cell surface and secreted proteins (Kessenbrock et al., 2010). Matrix metalloproteinases have been shown to play critical roles in a number of tumorigenic processes including cell proliferation, apoptosis, migration and invasion (Bissell et al., 2005). Non-malignant mouse mammary epithelial cells (SCp2) were engineered to express an inducible autoactivated version of MMP-3 (also known as stromelysin-1), which led to a disorganized and invasive phenotype in 3D culture, subsequent to cleavage of E-cadherin and release of transcriptionally-active β-catenin to the nucleus (normally found in adherens junctions) (Lochter et al., 1997). Of note, *MMP3* has been shown to be a transactivation target for mutant p53, in cooperation with oncogenic Ras (Buganim et al., 2010). Intriguingly, using this same system MMP-3 expression was later shown to induce genomic instability in a strikingly non-random fashion (preferential deletion/amplification of certain

chromosomal regions) by regulating the alternative splicing of Rac1 to Rac1b, the prominent isoform found in breast cancer (Radisky et al., 2005). MMP-3 is not the only protease that can initiate this pathway, as MMP-9 (although not MMP-2) could substitute for MMP-3 (Radisky et al., 2005).

In fact, MMP-9 has also been shown to profoundly influence acinar morphogenesis in the 3D culture system. Activated MMP-9 can disrupt polarity and re-initiate proliferation in non-tumorigenic MCF10A cells, disrupting acinar morphogenesis (Beliveau et al., 2010). Additionally, using two cell lines derived from the same patient, S1 (non-tumorigenic) and T4-2 (tumorigenic) (Briand et al., 1987; Weaver et al., 1997), it has been elegantly demonstrated that not only is activated MMP-9 sufficient to disrupt acinar morphogenesis when expressed in S1 cells, but MMP-9 is necessary to maintain the malignant state, as inhibition or downregulation of MMP-9 was sufficient to revert disorganized T4-2 cells to a more normal acinar-like morphology (Beliveau et al., 2010). T4-2 cells, which are highly tumorigenic in immunocompromised mice, exhibited dramatically reduced tumor growth *in vivo* upon MMP-9 downregulation by RNAi, supporting the notion that 3D phenotype can accurately predict tumorigenicity (Beliveau et al., 2010). Highlighting the dynamic reciprocity mentioned earlier, it was demonstrated that MMP-9 can cleave all three subunits of laminin-111, rearranging tissue architecture, altering signal transduction through the Raf/MEK/Erk pathway, which in turn regulates the levels of MMP-9 (Beliveau et al., 2010).

The ADAM (A Disintegrin And Metalloproteinase) family of proteins is a related group of peptidases that is critical for the shedding of a number of growth factors (Kenny and Bissell, 2007). Similar to the phenotypic reversion observed upon MMP-9 inhibition, when ADAM-17 (also known as TACE-1) was depleted from T4-2 cells using RNAi, these cells were able to form

acinar-like structures in a 3D microenvironment. This reversion was dependent upon the ability of ADAM17 to cleave, and thus shed, TGF-α and amphiregulin. These growth factors could then signal through the epidermal growth factor receptor (EGFR) to amplify the mitogen-activated kinase (MAPK, also known as Raf/MEK/ERK) pathway (Kenny and Bissell, 2007).

However, the relationship between MMPs and tissue architecture is not as clear cut is it may appear. Matrix metalloproteinases are endogenously controlled by tissue inhibitors of metalloproteinases (TIMPs). Although two MMPs can disrupt acinar morphogenesis, overexpression of TIMP-1, a natural inhibitor of MMPs, is sufficient to disrupt MCF10A acinar morphogenesis (Jung et al., 2006; Liu et al., 2005). While this may appear contradictory, in fact it rather points to the importance of the tight regulation for the levels/activity of MMPs in normal cells and highlights the dysregulation that occurs during tumorigenesis.

Further illustrating the intricate connection between the extracellular microenvironment and tissue architecture, it has been observed that antibodies directed against fibronectin, a key component of the ECM, can phenotypically revert tumorigenic T4-2 cells in 3D culture. These cells which are normally highly disorganized, formed a hollow lumen with proper basal polarization of integrin β4, demonstrating the dominance of the microenvironment (Sandal et al., 2007). The question still remains, how do microenvironmental changes modulate gene expression to disrupt acinar morphogenesis in 3D culture? The answer, to which we have already alluded, is that the ECM can transmit biochemical as well as biomechanical signals to a cell, which can then influence tissue architecture.

Cell Adhesion Molecules

Cell-matrix and cell-cell adhesion is largely mediated by the integrin family, but it is important to remember that these proteins also impinge upon multiple signal transduction pathways that affect various cellular processes. Integrins are heterodimeric transmembrane proteins, each is composed of a single α and β subunit from which they derive their names, and a number of integrins have been implicated in tumorigenesis (Gilcrease, 2007; Wang et al., 2002; Wang et al., 1998; Weaver et al., 1997). Altered levels/localization of integrins α2, α3, α6, β1 and β4 have all been reported in breast cancer (Weaver et al., 1997).

Using the HMT-3522 breast cancer progression series (described above), it was noted that tumorigenic T4-2 cells express much higher levels of integrin β1 compared to their non-tumorigenic counterparts (S1 cells). When integrin β1 was inhibited by function-blocking antibodies, T4-2 cells underwent a phenotypic reversion in 3D culture (Weaver et al., 1997). It was subsequently discovered that an intimate relationship exists between the levels of integrin β1 and the epidermal growth factor receptor (EGFR) in 3D culture, as each protein can regulate the levels of the other, although this cross-modulation is not present in 2D monolayers (Wang et al., 1998). A number of other breast tumor cell lines have now been shown to undergo phenotypic reversions in response to integrin β1 inhibition when combined with either a phosphatidylinositol 3-kinase (PI3K) inhibitor, a mitogen-activated protein kinase (MAPK) inhibitor or E-cadherin re-expression (Wang et al., 2002).

As mentioned previously, integrins are composed of an α and β subunit, and integrin α2 most commonly pairs with integrin β1. Re-expression of integrin α2 into a tumorigenic mouse mammary epithelial cell line that no longer expressed integrin α2 resulted in a phenotypic

reversion in these cells, restoring their ability to form acinar-like structures in a laminin-rich ECM (Zutter et al., 1995). This seemingly paradoxical result, similar to the observed roles of MMPs in 3D culture, may reflect cell line dependent differences in response to integrin β1 or perhaps integrin β1 is pairing with a different alpha subunit (i.e. α1, α3, α4, α5, α6) in these other breast cancer cell lines to mediate its oncogenic effects.

The story with integrin β4 is no less complex. Integrin β4 almost exclusively associates with integrin α6 and functions as the primary receptor for laminin-5 (laminin-332) (Guo and Giancotti, 2004). Tumors often express high levels of integrin β4, which may have prognostic impact (Lu et al., 2008; Weaver et al., 2002). A large body of literature has linked the α6β4 integrin to multiple aspects of tumorigenesis including increased survival, angiogenesis and invasion (Lipscomb et al., 2005; Nikolopoulos et al., 2004; Shaw et al., 1997). In fact, β4 integrin has been shown to augment ErbB2-mediated breast tumorigenesis *in vivo* (Guo et al., 2006). However, the role that α6β4 integrin plays in tumorigenesis remains controversial. Other studies have demonstrated a reduced expression of integrin β4 in tumors and integrin β4 expression has been shown to mediate multiple tumor suppressive mechanisms in cell culture studies (i.e. apoptosis in a wild-type p53 dependent manner, as well as cause growth arrest mediated by p21) (Bachelder et al., 1999; Clarke et al., 1995; Davis et al., 2001; He et al., 2008).

Fitting with this theme, the role of integrin β4 in acinar morphogenesis is still up for debate. It has been demonstrated that downregulation of integrin β4 from non-tumorigenic S1 cells can disrupt their morphology in 3D culture (Weaver et al., 2002; Weaver et al., 1997). However, it has also been demonstrated that downregulation of integrin β4 from disorganized or invasive breast cancer cells can partially revert their morphology (Dutta and Shaw, 2008; Gabarra et al., 2010; Lipscomb et al., 2005). As the different cell lines employed in these studies

differ in their p53 mutational status, it is tempting to speculate that this may impact the function of integrin β4 in tumorigenesis (this theme will be explored further in Chapter 3).

In addition to the integrin family of proteins, a number of other adhesion molecules have been implicated in acinar morphogenesis. Dystroglycan, a non-integrin laminin receptor, has been shown to be downregulated in a subset of mammary tumors. Re-expression of dystroglycan in T4-2 cells, which normally express very low levels, was sufficient to restore proper apicobasal polarization and acinar morphogenesis in response to culture in a laminin-rich ECM (Muschler et al., 2002).

E-cadherin is a critical component of adherens junctions, which are important for cell-cell adhesion among epithelial cells, and is commonly lost in invasive mammary lobular carcinomas (Meiners et al., 1998). Acinar morphogenesis was severely disrupted in non-tumorigenic mammary epithelial cells when E-cadherin was inhibited by function-blocking antibodies or downregulated by means of RNAi (Fournier et al., 2009). Likewise, tumorigenic breast cell lines could be partially phenotypically reverted by re-expression of E-cadherin (Meiners et al., 1998), and could completely revert breast cancer cells to an acinar-like morphology when combined with inhibition of PI3K, MAPK or integrin β1 (Wang et al., 2002). Likewise, CEACAM1, a critical cell adhesion molecule expressed in a number of epithelial cell types, is frequently lost in tumors and has been demonstrated to be necessary for mammary lumen formation. When CEACAM1 was downregulated by siRNA in MCF10F cells (derived from the same patient as MCF10A cells), they could no longer form a hollow lumen and when CEACAM1 was exogenously expressed in MCF7 breast cancer cells, which normally cannot form a hollow lumen and which basally lack CEACAM1 expression, these cells could now form a hollow lumen (Huang et al., 1999).

In addition to cell adhesion molecules, one of the primary ways that signals are transmitted from the extracellular microenvironment is through receptor tyrosine kinases, a number of which have now been implicated in acinar morphogenesis.

Receptor Tyrosine Kinases

Modulation of a number of receptor tyrosine kinases (RTKs) can disrupt acinar morphogenesis in 3D culture. The human epidermal growth factor receptor 2, HER2/neu (also known as ErbB2), is commonly amplified in breast cancer and associated with a very poor prognosis (Muthuswamy et al., 2001; Sorlie et al., 2001). When MCF10A cells were engineered to express an inducible version of ErbB2, activation of this receptor in growth-arrested, polarized acinar structures resulted in a re-initiation of proliferation, disruption of apicobasal polarity, with consequent luminal filling (Muthuswamy et al., 2001). Interestingly, overexpression of the human epidermal growth factor receptor 1, EGFR (also known as ErbB1) can prevent anoikis, however this was not sufficient to disrupt acinar morphogenesis in MCF10A cells (Muthuswamy et al., 2001; Reginato et al., 2003). On the other hand, it has now been elegantly demonstrated that by inhibiting the activity of EGFR (with specific inhibitors, function-blocking antibodies or inhibiting the shedding of its ligands as described above) in disorganized, tumorigenic T4-2 cells this is sufficient to lead to a phenotypic reversion to a more acinar-like morphology in 3D culture (Kenny and Bissell, 2007; Wang et al., 2002; Wang et al., 1998). Intriguingly, EGFR is a well-recognized transcriptional target of tumor-derived p53 mutants (Ludes-Meyers et al., 1996).

Elevated activity of the insulin-like growth factor 1 receptor (IGF1R) has also been shown to induce a luminal filling phenotype in MCF10A cells grown in 3D culture (Irie et al., 2005; Irie et al., 2010; Litzenburger et al., 2009). The insulin-like growth factor (IGF) family

comprises two ligands (IGF-1 and IGF-2), two cell surface receptors (IGF1R and IGF2R), in addition to a number of binding proteins (IGFBP1-6). Expression of the IGF family members is often altered in human cancers, including breast cancer (Litzenburger et al., 2009). Of particular interest to this work, a number of IGF family members are transactivation targets of mutant p53, including IGF1R (Table 1.1). Similarly, elevated activity of the fibroblast growth factor receptor 1 (FGFR1) is commonly observed in invasive lobular carcinoma of the breast. MCF10A cells that had been engineered to activate FGFR1 in an inducible manner, exhibit an inability to undergo luminal clearance upon FGFR1 activation, in addition to displaying altered apicobasal polarization. FGFR1 signals downstream to the mitogen-activated protein kinase (MAPK) pathway and the phenotypic alterations in 3D culture were shown to be dependent on the activity of this downstream pathway (Xian et al., 2009).

Colony-stimulating factors (CSF) have been most widely studied for their role in promoting the proliferation and survival of hematopoietic cells; however, the CSF-1/CSF-1R pair has also been implicated in outgrowth of the human mammary gland and shown to be elevated in breast tumors (Dai et al., 2002; Kacinski et al., 1991). Colony-stimulating factor 1 receptor (CSF-1R) encodes a tyrosine kinase transmembrane receptor and expression of a constitutively active version of CSF-1R, or co-expression of CSF-1/CSF-1R, profoundly disrupted acinar morphogenesis in MCF10A cells, by preventing luminal clearance and disrupting cell-cell adhesion (Wrobel et al., 2004). As a consequence of CSF-1R hyperactivation, E-cadherin was progressively lost from the cell membrane, which may account for the discohesive phenotype observed in 3D culture. Mechanistically, CSF-1R signals downstream to Src, a proto-oncogenic tyrosine kinase, and Src activation was shown to be necessary for the phenotypic alterations induced by CSF-1R (Wrobel et al., 2004). In fact, Src

overexpression alone was subsequently demonstrated to be sufficient to disrupt acinar morphogenesis of MCF10A cells in 3D culture (Reginato et al., 2005). Conversely, c-Met, the key cellular receptor for hepatocyte growth factor (HGF), is another receptor tyrosine kinase that signals downstream to Src. Expression of c-Met in conjunction with HGF resulted in a similar activation of Src to that of CSF-1R, which led to the formation of branched tubules in 3D culture. However, unlike the severely disrupted phenotype with hyperactive CSF-1R, structures formed with hyperactive c-Met retain a hollow lumen and proper adherens junctions (Wrobel et al., 2004). This highlights the fact that although many of the same signaling molecules are activated in response to different stimuli, disparate receptor activation can result in markedly different phenotypic changes.

While not receptor tyrosine kinases themselves, protein tyrosine phosphatases normally suppress the activity of a variety of RTKs. Recently, protein tyrosine phosphatase non-receptor type 12 (PTPN12), which regulates the activity of EGFR and HER2/neu, among others, has been implicated in disruption of mammary tissue architecture. Depletion of PTPN12 in MCF10A cells using RNAi resulted in hyperactivation of a number of RTKs (HER2 and EGFR, primarily), which severely compromised acinar morphogenesis in 3D culture, bypassing proliferative suppression and inducing a luminal filling phenotype, in addition to structures with altered shapes (Sun et al., 2011).

Interestingly, extracellular factors, cell adhesion molecules and receptor tyrosine kinases impinge on similar, and often overlapping, intracellular signaling cascades.

Intracellular Signal Transduction

The phosphatidylinositol 3-kinase (PI3K) pathway, which signals to Akt and mTOR, promotes cell proliferation and survival. Not surprisingly, it is one of the most commonly altered pathways in human cancer (Cantley, 2002). The two most commonly mutated genes are *PIK3CA* and *PTEN*. *PIK3CA* encodes the p110α catalytic subunit for phosphatidylinositol 3-kinase, which similar to other mutated oncogenes, commonly incurs gain-of-function missense mutations on hot-spot residues (i.e. E542K, E545K and H1047R) leading to elevated activity (Gymnopoulos et al., 2007). Functional loss of phosphatase and tensin homolog (PTEN), an endogenous inhibitor of PI3K that signals to convert phosphatidylinositol (3,4,5)-trisphosphate (PIP3) back to phosphatidylinositol (4,5)-bisphosphate (PIP2), commonly occurs in tumorigenesis (Simpson and Parsons, 2001). Multiple studies have implicated this pathway as playing a significant role in acinar morphogenesis.

When two of the most commonly occurring tumor-derived PIK3CA mutants (E545K and H1047R) were introduced into MCF10A cells, this resulted in highly proliferative, multi-acinar 3D structures (Isakoff et al., 2005). Importantly, treatment with rapamycin, an inhibitor of mTOR, reversed these phenotypic effects, suggesting that the canonical Akt/mTOR pathway was mediating the effects of mutant PIK3CA on tissue architecture (Isakoff et al., 2005). Similarly, expression of a conditionally activated version of the serine/threonine kinase Akt1 was sufficient to disrupt MCF10A acinar morphogenesis (Debnath et al., 2003b). Likewise, Akt signaling was shown to be necessary for the disrupted phenotype induced by IGF1R; however, a specific role for Akt2 was noted in this context (Irie et al., 2005). Downregulation of PTEN has also been examined in the 3D culture system, although with somewhat contradictory results. RNAi-mediated depletion of PTEN from non-tumorigenic S1 cells was sufficient to disrupt acinar morphogenesis (Fournier et al., 2009); however, homozygous deletion of both PTEN alleles

from MCF10A cells prevented anoikis, but did not disrupt acinar morphogenesis (Vitolo et al., 2009). A number of reports now exist that demonstrate the importance of the PI3K pathway in maintaining the malignant state. Specific inhibition of PI3K has been shown to phenotypically revert a number of tumorigenic breast cell lines, either alone or in combination with other inhibitors (Beliveau et al., 2010; Liu et al., 2004b; Wang et al., 2002).

In addition to the PI3K pathway, the Raf/MEK/ERK pathway has been shown to be deeply involved with the process of acinar morphogenesis. Inducible expression of Raf, a mitogen-activated kinase kinase kinase that phosphorylates and activates MEK-1/2, in growth-arrested acinar structures was sufficient to re-initiate proliferation and lead to a disorganized, apolar 3D phenotype (Beliveau et al., 2010). Similarly, inducible expression of an oncogenic variant of B-Raf (V600E) in growth-arrested MCF10A acinar-like structures, profoundly disrupted their 3D morphology, with a re-initiation of proliferation, disorganization and invasion into the surrounding matrix (Herr et al., 2011). Interestingly, acinar-like structures could be recovered upon shutting off expression of oncogenic B-Raf, illustrating the plasticity of the malignant phenotype (Herr et al., 2011). Furthermore, by using specific MEK inhibitors, it has been demonstrated that this pathway is necessary to maintain the malignant state of many tumorigenic breast cell lines. Relating to previously mentioned work, the Raf/MEK/ERK pathway is downstream of EGF and integrin β1, and this pathway controls the expression of MMP-9 (Beliveau et al., 2010; Wang et al., 2002; Wang et al., 1998).

Rap1 is a member of the Ras superfamily of small GTPases and plays a critical role in mediating signals from a number of cytokines, growth factors and adhesion molecules. Like all regulatory GTPases, Rap1 can exist in either an inactive guanine nucleotide diphosphate (GDP)-bound state or an activated guanine nucleotide triphosphate (GTP)-bound state. Activity is

elevated by specific guanine nucleotide exchange factors (GEFs), which promote dissociation of bound GDP thus allowing the protein to bind a new GTP. Conversely, GTPase activating proteins (GAPs) promote the intrinsic GTPase activity of the protein leading to hydrolysis of the bound GTP to GDP, therefore cycling the protein back to its inactive state. Of particular interest to this work, Rap1 is anchored to lipid membranes through the covalent attachment of geranylgeranyl lipid moieties to the C-terminus of the protein, which is necessary for proper functioning (Stork and Dillon, 2005). Using the HMT-3522 breast cancer progression series, it was observed that while Rap1 levels were not dramatically altered between non-tumorigenic S1 cells and tumorigenic T4-2 cells, the levels of GTP-bound Rap1 was much higher in disorganized T4-2 clusters of cells. Interestingly, this distinction was only evident when assayed from 3D culture (Itoh et al., 2007). Inhibition of Rap1 activity in T4-2 cells using a dominant-negative version of Rap1 (S17N), restored proper apicobasal polarization and lumen formation (Itoh et al., 2007). Interestingly, the ability of EGFR and MAPK inhibition to phenotypically revert malignant T4-2 cells to a more acinar-like morphology with correct polarity and a hollow lumen has been shown to be dependent upon regulation of Rap1 activity, as this could be prevented by expression of dominant-active Rap1 (Itoh et al., 2007).

While the primary insult(s) that disrupts acinar morphogenesis in many of the above examples appears to be either escape from proliferative suppression, inhibition of cell death or a combination of the two, a group of cytoplasmic signaling proteins have now been described the principal function of which is to govern apicobasal polarity. A number of proteins have been implicated in determining apicobasal polarity in *Drosophila melanogaster* and many of these proteins are conserved among vertebrates. These include the partitioning defective 3 (Par3)/Par6/atypical protein kinase C (aPKC) complex, Crumbs/PALS/PATJ complex and the

scribble (Scrib)/lethal giant larval (Lgl)/discs large (Dlg) proteins (Zhan et al., 2008). Since formation of apicobasal polarity is an essential step in proper acinar development, many of these proteins have now been explored in the context of the 3D laminin-rich ECM system.

Loss of Scrib, the mammalian ortholog of Scribble, is frequently observed in invasive mammary lobular carcinoma (Zhan et al., 2008). Depletion of Scrib using RNAi from MCF10A cells resulted in disrupted polarity in 3D culture with consequent luminal filling. Loss of Scrib not only affected ductal morphogenesis, but also cooperated with Myc to induce murine mammary tumors (Zhan et al., 2008). Likewise, PKCζ, a member of the Par complex, has been shown to be critical for proper acinar development. Using a non-phosphorylatable mutant of PKCζ, it was demonstrated that activity of this protein is essential for proper apicobasal polarization of MCF10A cells in 3D culture (Whyte et al., 2010). Additionally, expression of a kinase-dead version of PKCζ led to hyperproliferative, multi-acinar structures, suggesting that, like Scrib, the Par polarity complex may function as a tumor suppressor in breast cancer (Whyte et al., 2010). However, the Par6/aPKC complex has been shown to interact with ErbB2 and a dominant-negative version of Par6 can actually rescue the phenotypic effects of ErbB2 overexpression on MCF10A in 3D culture (Aranda et al., 2006).

Nuclear Proteins

One of the most famous genes associated with familial breast cancer is the breast cancer 1, early onset (*BRCA1*) gene, which functions as a tumor suppressor largely by maintaining genomic stability. In fact, mutations in the *BRCA1* gene may account for up to half of all inherited breast cancers (Furuta et al., 2005). Depletion of BRCA1 (the protein encoded by *BRCA1*) from MCF10A cells using RNAi severely disrupted acinar morphogenesis in 3D

culture. Very interestingly, proper acinar development could be rescued if BRCA1-deficient cells were supplemented with conditioned medium taken from normally developing MCF10A cells in 3D culture, suggesting that there are certain critical factors being secreted by BRCA1-positive cells that are necessary for proper acinar morphogenesis (Furuta et al., 2005).

A screen for copy number changes in breast cancer identified an amplified region on 11q22, containing the Yes-Associated Protein (YAP), using a p53/BRCA1-deficient model of mammary carcinogenesis. Expression of YAP in MCF10A cells led to highly invasive structures when grown in a laminin-rich ECM (Overholtzer et al., 2006). Interestingly, YAP has previously been shown to physically interact with p73, enhance its transcriptional activity and promote apoptosis (Strano et al., 2001). Thus, YAP appears to play both tumor suppressive and oncogenic roles depending on cellular context.

Homeobox (Hox) genes encode proteins that function as "master regulator" transcription factors to direct many developmental processes. In addition to their functions during embryonic development, it has now been observed that expression of Hox proteins is often altered in tumorigenic tissue. HoxD10 expression is often reduced in breast cancer and re-expression of this critical transcription factor in highly malignant MDA-231 breast cancer cells was sufficient to induce a phenotypic reversion to a growth-arrested, polar acinar-like morphology in 3D culture (Carrio et al., 2005).

Of particular interest to this work, it was recently demonstrated that expression of three of the most common hot-spot p53 mutants (R175H, R248W, R273H) could disrupt MCF10A acinar morphogenesis, while expression of a different hot-spot mutant (G245S) had no effect on 3D morphology (Zhang et al., 2011). In order to address potential dominant-negative effects of

these p53 mutants, this group engineered MCF10A cells depleted of wild-type p53 using RNAi and demonstrated that wild-type p53 is not required for acinar development, although they did note a mild effect on luminal clearance upon wild-type p53 depletion. In fact, expression of tumor-derived p53 mutants in the background of wild-type p53 depletion amplified the luminal filling phenotype (Zhang et al., 2011). However, it is important to keep in mind that despite the strong suggestion of gain-of-function effects of mutant p53 in this 3D system, it is not possible to rule out a dominant-negative effect of the tumor-derived mutants on remaining wild-type p53 even after RNAi-mediated depletion.

3D culture and metabolism

While a few studies have examined metabolic differences between cells grown under 2D or 3D culture conditions, this is an area that is just beginning to be widely recognized. For example, it has long been known that transformed cells grown in 3D culture exhibit lower levels of glucose uptake compared to the same cells grown as monolayers (Bissell et al., 1977). Additionally, one of the key biological processes regulated during acinar morphogenesis is lipid metabolism (Fournier and Martin, 2006; Fournier et al., 2006), which may offer important insight into this work. This is particularly important for tissue-specific functions, such as lactation (Rodriguez-Cruz et al., 2006; Rudolph et al., 2007). As noted above, one mechanism by which mammary epithelial cells can clear cells from their lumina is through depletion of ATP in cells detached from the ECM, the importance of which becomes apparent when apoptosis is inhibited. In fact, HER2/neu overexpression in MCF10A cells not only inhibits luminal apoptosis, but also bypasses this backup mechanism by increasing glucose uptake and therefore ATP generation, allowing cells to inappropriately collect in the lumen (Schafer et al., 2009).

In addition, the activity of a number of transcription factors has been shown to be highly regulated by culture in a 2D versus a 3D environment. Of particular interest to this work, the activity of the SREBP1 transcription factor (as measured by binding affinity to its cognate response element) was shown to be approximately 60 times higher in 3D culture compared to 2D culture (Dozmorov et al., 2008).

The History of Cholesterol

The isolation of cholesterol dates back nearly two hundred years to Michel Chevreul, a French chemist, who first proposed the name "cholesterine" (derived from Greek: *chole*=bile and *stereos*=solid), as it had first been identified in gallstones (Vance and Van den Bosch, 2000). The molecule was later renamed cholesterol and was shown to be a vital component of all animal cells. Heinrich Wieland and Adolf Windaus shared the 1928 Nobel Prize in Chemistry for elucidating the complex structure of cholesterol (an interesting historical note is that the structure for which they shared the Nobel Prize was not entirely correct, the structure we now know to be cholesterol was not solved until 1932) (Vance and Van den Bosch, 2000). As noted above, all animal cells require cholesterol, which they can acquire through two mechanisms: *de novo* biosynthesis and uptake of low density lipoproteins (LDL) that contain esterified cholesterol (Vance and Van den Bosch, 2000).

Rudolph Schoenheimer, first in Berlin and then at Columbia University, pioneered the technique of isotope-labeling to study biosynthetic processes which would be used to elucidate the complex process of cholesterol biosynthesis. In fact, Schoenheimer's groundbreaking work paved the way for at least three later Nobel Prizes as he began the work of elucidating the cholesterol biosynthetic pathway, also known as the mevalonate pathway, which earned Konrad

Bloch the Nobel Prize in Physiology or Medicine in 1964, and was the first to demonstrate the feedback regulation of cholesterol biosynthesis, which led Michael Brown and Joe Goldstein to their Nobel Prize in Physiology or Medicine in 1985 (Vance and Van den Bosch, 2000). It was subsequently shown that nearly all mammalian cells have the ability to carry out *de novo* cholesterol biosynthesis (Siperstein, 1970).

The Mevalonate Pathway (isoprenoid/sterol biosynthetic pathway)

The biosynthesis of cholesterol is achieved through a series of enzymatic events involving over 30 different enzymes and numerous cofactors (Vance and Van den Bosch, 2000). This pathway also produces a number of biomolecules that are important for a variety of cellular processes including cell growth, protein glycosylation, protein prenylation and mitochondrial electron transport (Waterham, 2006). The key enzymatic steps in the mevalonate pathway are detailed below and depicted in Figure 2.S3 (Brown and Goldstein, 1980; Goldstein and Brown, 1990; Waterham, 2006).

The mevalonate pathway begins with the two carbon acetyl-CoA, which combines with another molecule of acetyl-CoA to become acetoacetyl-CoA through the action of cytosolic acetyl-CoA acetyltransferase (encoded by *ACAT2*). Condensation of acetoacetyl-CoA with another molecule of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is catalyzed by cytosolic HMG-CoA synthase (encoded by *HMGCS1*). HMG-CoA is subsequently reduced to mevalonate through two molecules of NADPH. This reaction is catalyzed by an endoplasmic reticulum (ER)-bound enzyme, HMG-CoA reductase (encoded by *HMGCR*), which is the rate limiting step in the mevalonate pathway. Mevalonate is then phosphorylated by two cytosolic enzymes mevalonate kinase and phoshomevalonate kinase (encoded by *MVK* and

PMVK, respectively). Mevalonate decarboxylase (encoded by *MVD*) then catalyzes the decarboxylation of 5-pyrophosphate mevalonate to isopentenyl pyrophosphate. This is the basic 5-carbon isoprene unit used for all subsequent isoprenoid synthesis. Cytosolic isopentenyl pyrophosphate isomerase (encoded by *IDI1*) interconverts isopentenyl pyrophosphate (IPP) with dimethylallyl pyrophosphate (DMAPP). One molecule of IPP combines with one molecule of DMAPP in a "head-to-tail" condensation to form geranyl pyrophosphate, which subsequently combines with another molecule of IPP in another "head-to-tail" condensation reaction catalyzed by farnesyl pyrophosphate synthase (encoded by *FDPS*) to form 15-carbon farnesyl pyrophosphate (FPP). Farnesyl pyrophosphate represents the major branchpoint in the mevalonate pathway as it can continue on to cholesterol or be diverted into a number of biologically active metabolites such as geranylgeranyl pyrophosphate, dolichol and ubiquinone. The importance of these offshoots will be detailed below.

If cholesterologenesis is to continue, squalene synthase (encoded by *FDFT1*) combines two molecules of FPP in a "head-to-head" condensation reaction to form 30-carbon squalene, the first committed intermediate in the sterol biosynthetic pathway. Squalene epoxidase (encoded by *SQLE*) next converts squalene to 2,3-oxidosqualene, a reaction which requires O_2 and NADPH. 2,3-oxidosqualene is then cyclized to lanosterol, catalyzed by lanosterol synthase (encoded by *LSS*). Lanosterol is then converted to cholesterol through at least eight additional enzymatic steps, the order of which is thought to be tissue-dependent. The end-product of the canonical mevalonate pathway is the 27-carbon cholesterol molecule, which can subsequently be incorporated into membranes or be used for vitamin D or steroid hormone synthesis.

Non-sterol products of the mevalonate pathway

While the mevalonate pathway has been studied most extensively in relation to cholesterol production, particularly in the liver, it is important to keep in mind that nearly all mammalian cells have this pathway intact and have the ability to generate cholesterol *de novo*. This pathway is not only responsible for cholesterol production, but for many mevalonate-derived intermediate metabolites that have diverse biological functions. Some of the key biomolecules produced by the mevalonate pathway will reviewed below.

As mentioned above, farnesyl pyrophosphate (FPP) is a critical branch point in the mevalonate pathway, as it can give rise to squalene and thus cholesterol, or be diverted to geranylgeranyl pyrophosphate on route to a number of important non-sterol products (Mo and Elson, 2004). Additionally, farnesyl pyrophosphate and geranylgeranyl pyrophosphate can be post-translationally attached to proteins on their C-terminus, serving to anchor these proteins to lipid membranes (Basso et al., 2006). Protein prenylation, the term that encompasses farnesylation and geranylgeranylation of proteins, can occur through the action of farnesyl transferase or geranylgeranyl transferases.

These lipid moieties are covalently attached to cysteine residues embedded in specific sequences in the extreme C-terminal regions of target proteins. The cysteine residue in CaaX motifs[3] is the target for farnesylation by farnesyl transferase. Geranylgeranylation can occur through the activity of geranylgeranyl transferase-1 (GGTase-1) or geranylgeranyl transferase-2 (GGTase-2). GGTase-1 targets proteins with a C-terminal leucine residue (CaaL motifs)[4],

[3] C=cysteine, A=aliphatic amino acid (I, L, V, A or P), X=any amino acid

[4] C=cysteine, A=aliphatic amino acid (I, L, V, A or P), L=leucine

whereas GGTase-2 targets proteins with two C-terminal cysteines (XXCC, XCXC or CCXX)[5] (Basso et al., 2006). While a number of target proteins can be either farnesylated or geranylgeranylated, a subset of target proteins are specific for one modification (Maurer-Stroh et al., 2007). For example, H-Ras is farnesylated and RhoA is geranylgeranylated (Casey et al., 1989; Fried, 2008; Yoshida et al., 1991). In addition to these famous targets, other proteins targeted by prenylation include nearly all small G-proteins in addition to proteins such as nuclear lamins (Basso et al., 2006).

Another form of post-translational modification is also regulated by the mevalonate pathway, as dolichol is a critical determinant in N-linked glycosylation of proteins (Graaf et al., 2004). Additionally, the mevalonate pathway produces ubiquinone (also known as coenzyme Q_{10}), a vital component of the mitochondrial electron transport chain, and isopentyladenine, a key constituent of transfer RNAs (tRNAs) (Brown and Goldstein, 1980; Fears, 1981).

Regulation of the mevalonate pathway

Since the early experiments by Schoenheimer in the 1930's, later confirmed by a number of groups in the 1950's-1960's, it has been demonstrated that cholesterol biosynthesis is tightly regulated to maintain homeostasis (Edwards et al., 2000; Vance and Van den Bosch, 2000). The two major mechanisms controlling cholesterol homeostasis are the levels/activity of enzymes involved in *de novo* biosynthesis and levels of the low density lipoprotein receptor (LDLR), responsible for uptake of cholesterol in the form of plasma LDL (Goldstein and Brown, 1990). When adequate levels of sterols are present in cells, the levels of LDLR and the cascade of enzymes involved in cholesterologenesis are kept at a relatively constant level; however, upon

[5] C=cysteine, X=any amino acid

conditions of sterol deprivation, the expression of the LDL receptor and nearly all enzymes in the mevalonate pathway are rapidly induced (Bengoechea-Alonso and Ericsson, 2007).

This finely tuned regulation has been shown to occur at multiple levels, including degradation of the rate-limiting enzyme HMG-CoA reductase, but is thought to occur primarily at the level of transcription (Sato, 2010). A family of transcription factors dubbed the sterol regulatory element binding proteins (SREBPs) was found to regulate the expression of nearly every enzyme in the cholesterol (as well as the fatty acid) biosynthetic pathways (Goldstein et al., 2006; Hua et al., 1993; Wang et al., 1994). SREBPs are basic-helix-loop-helix-leucine-zipper (bHLH-leucine zipper) transcription factors; however, they are unique members of this class of transcription factors in at least two important respects. First, they are synthesized as inactive precursors (approximately 1150 amino acids), which are bound in the endoplasmic reticulum (ER) (Eberle et al., 2004). These precursor SREBPs are bound in a hairpin fashion through the ER membranes with both the N-terminal transcription factor domains and C-terminal regulatory domains facing the cytosol. A short loop projecting into the ER lumen connects the two membrane-spanning helices (Goldstein et al., 2006). When levels of cellular sterols drop, SREBPs are transported to the Golgi apparatus where they undergo successive cleavage events to release N-terminal fragments of approximately 480 amino acids. These N-terminal (mature) SREBPs are subsequently transported into the nucleus where they can dimerize and function as sequence-specific transcription factors (Bengoechea-Alonso and Ericsson, 2007). Secondly, while other bHLH-leucine zipper transcription factors recognize E-boxes (5'-CANNTG-3')[6], SREBPs can recognize both E-boxes as well as sterol regulatory elements, represented as SRE-1

[6] A=adenine, T=thymine, C=cytosine, G=guanine, N=any nucleotide.

(5'-TCACNCCAC-3')[7] (Eberle et al., 2004; Hua et al., 1993; Wang et al., 1994). Both SREBP-1 and SREBP-2 conserve the critical amino acids in the bHLH-leucine zipper, implicated in DNA recognition by Max, another bHLH-leucine zipper transcription factor (Hua et al., 1993). This altered sequence-specificity has been shown to be due to a tyrosine substitution for a conserved arginine in the basic region, which allows the SREBPs to recognize the direct repeats instead of palindromic sequences (Osborne and Espenshade, 2009).

There are three known members of the SREBP family: SREBP-1a and SREBP-1c (encoded by the same gene *SREBF1*) and SREBP-2 (encoded by *SREBF2*). SREBP-1a and SREBP-1c use alternative transcriptional start sites, differing only in their first exon (Osborne, 2000). As the transactivation domain of SREBP-1 is largely contained in the first exon, SREBP-1c encodes significantly fewer acidic residues and is thus a much weaker transcriptional activator compared to SREBP-1a (Eberle et al., 2004). SREBP-1 and SREBP-2 share 47% homology overall and are 71% identical in the bHLH-leucine zipper region, yet interestingly they have been shown to preferentially transactivate distinct subsets of target genes (McPherson and Gauthier, 2004). SREBP-1a has been shown to be capable of transactivating nearly every key enzyme in the fatty acid and cholesterol biosynthetic pathways. Likewise, SREBP-2 can transactivate nearly every gene in both biosynthetic pathways, although it shows a strong preference for cholesterol biosynthetic enzymes. SREBP-1c, on the other hand, is very specific for fatty acid biosynthetic genes and as mentioned previously, is a much weaker transcriptional activator than either of the other family members. In order to function as transcription factors, SREBPs most commonly homo-dimerize. To complicate matters hetero-dimerization between isoforms has also been shown to occur, although the effect on target selectivity has not yet been

[7] A=adenine, T=thymine, C=cytosine, G=guanine, N=any nucleotide.

examined (Datta and Osborne, 2005). It has also been observed that the SREBPs are rather weak transcriptional activators on their own and most often require co-activators such as NF-Y or Sp1 for efficient transactivation (Osborne, 2000; Reed et al., 2008).

In normal cells, SREBP activation is tightly regulated by two proteins, the SREBP Cleavage Activating Protein (SCAP) and insulin-induced genes (INSIGs) (Brown and Goldstein, 2009). SCAP directly binds the C-terminus of SREBPs and this SCAP-SREBP complex is held in the ER by INSIG, which is anchored in the ER membrane. The interaction between SCAP and INSIGs is mediated by regulatory sterols. Thus when sterol levels in the cell fall, the interaction between SCAP and INSIG is alleviated and the SCAP-SREBP complex is transported to the Golgi apparatus in a COPII-dependent manner (Osborne and Espenshade, 2009). In the Golgi, the SREBPs are cleaved by two proteases bound within the Golgi-membrane. Site-1 protease (S1P) first cleaves the precursor SREBP within the luminal loop. Only then can the site-2 protease (S2P) cleave SREBP within the transmembrane segment, releasing the N-terminal mature SREBP, which is then translocated to the nucleus through a pathway that requires importin-β. Completing the feedback loop, mature SREBPs transactivate the gene encoding INSIG thus shutting off SREBP signaling once sufficient sterol levels are restored (Goldstein et al., 2006; Osborne and Espenshade, 2009).

Mevalonate pathway inhibitors

Cholesterol has long been associated with atherosclerosis and coronary heart disease (Tobert, 2003). Thus effective inhibitors for cholesterol biosynthesis were avidly pursued during the 1970s. In 1976, Endo and colleagues isolated a compound from a fermentation broth of *Penicillium citrinum*, which was shown to be a potent competitive inhibitor of HMG-CoA

reductase, the rate-limiting step in cholesterologenesis, which came to be known as compactin (also known as ML236B) (Brown and Goldstein, 1980). Four years later, a second HMG-CoA reductase inhibitor was isolated from a fermentation broth of *Aspergillus terreus*. This second compound was initially named mevinolin, but was later renamed lovastatin, and went on to become the first FDA-approved HMG-CoA reductase inhibitor in 1987 (Tobert, 2003).

The statin class of compounds are structurally similar and all function as competitive inhibitors to HMG-CoA reductase, competing for HMG-CoA binding (Tobert, 2003; Wong et al., 2002). Statins can be categorized into either a lipophilic or hydrophilic class. Whereas lipophilic statins exhibit no tissue selectivity and thus permeate multiple tissue types, hydrophilic statins are specifically designed to have a higher affinity for hepatic tissue (Tobert, 2003). While this is often seen as a benefit for treatment of hypercholesterolemia, as hepatic-selective statins exert fewer systemic side effects, lipophilic statins appear to have a much more potent anti-cancer effect on non-hepatic tissue, as will be discussed in more detail later.

In addition, a number of other inhibitors that perturb the mevalonate pathway are being explored. For example, a number of compounds which inhibit farnesyl transferase or geranylgeranyl transferase are under investigation as anti-cancer agents, although these have not shown much promise in early phase clinical trials (Mo and Elson, 2004). A recently described inhibitor that blocks SREBP activation, termed Fatostatin, may also show promise as an anti-cancer therapeutic (Kamisuki et al., 2009). In addition, Metformin, a first-line agent for type II diabetes, has also been shown to inhibit the mevalonate pathway (Nilsson et al., 2011). This is presumably due to its activation of AMP-activated protein kinase (AMPK), which inhibits the SREBP transcription factors (Li et al., 2011c). Interestingly, Metformin has previously been shown to specifically inhibit tumor growth in p53-deficient cell lines (Buzzai et al., 2007).

The Mevalonate Pathway and Cancer

A number of different tumor types, including breast cancer, exhibit elevated or dysregulated activity of the mevalonate pathway (Koyuturk et al., 2007; Wong et al., 2002). In fact, a number of studies have suggested that malignant cells are more highly dependent on the continuous availability of metabolites produced by the mevalonate pathway than their non-malignant counterparts (Buchwald, 1992; Larsson, 1996). Additionally, many tumor cells have lost the feedback inhibition normally present in the mevalonate pathway (Buchwald, 1992; Siperstein, 1970). The mevalonate pathway has been demonstrated to be necessary for DNA synthesis (Langan and Volpe, 1986; Quesney-Huneeus et al., 1979) and a high levels of multiple enzymes in this pathway have been shown to have prognostic significance in breast cancer (Clendening et al., 2010). Ectopic expression of HMG-CoA reductase or supplementation with additional mevalonate has been shown to increase tumorigenicity in mouse models (Clendening et al., 2010; Duncan et al., 2004). In accordance with these findings, HMG-CoA reductase inhibitors have been highly effective in pre-clinical models of tumorigenesis, both in cell culture and *in vivo* (Campbell et al., 2006; Cao et al., 2011; Denoyelle et al., 2001; Kidera et al., 2010; Lim et al., 2009; Sanchez et al., 2008; Shachaf et al., 2007; Shibata et al., 2004; Shibata et al., 2003; Sutter et al., 2005; Zhong et al., 2005).

There is also a growing body of literature suggesting that inhibition of the mevalonate pathway, particularly with HMG-CoA reductase inhibitors, may reduce tumor growth or even prevent tumor formation. Multiple retrospective analyses have observed dramatically decreased rates of breast, colon, lung and prostate cancers in patients prescribed statins for hypercholesterolemia (Blais et al., 2000; Katz et al., 2005; Khurana et al., 2007a; Khurana et al., 2007b; Poynter et al., 2005). There is particularly strong evidence for lipophilic statins as

potential chemopreventive agents in breast cancer (Ahern et al., 2011; Blais et al., 2000; Cauley et al., 2003; Garwood et al., 2010; Kumar et al., 2008; Kumar et al., 2006; Kwan et al., 2008; Pedersen et al., 2000; Prowell et al., 2006; Stein E A, 1993). However, it should be noted that the anti-cancer activity of HMG-CoA reductase inhibitors is still hotly debated, as a number of meta-analyses have failed to show any correlation between statin use and tumor incidence (Baigent et al., 2005; Browning and Martin, 2007). One possibility, which will be raised in Chapter 2, is that statins may target a specific molecular subtype (or subtypes) of tumors and this anti-cancer effect is diluted by grouping all patients together for epidemiologic purposes. In relation to this work, it will be particularly interesting to examine the rate of *TP53* mutations in tumors that develop in statin users.

Concluding Remarks

p53 plays a pivotal role in many aspects of tumor biology, thus it is not surprising that it is mutated in the majority of human malignancies (Vogelstein et al., 2000). It is now clear that many tumor-derived p53 missense mutants not only reflect a loss of wild-type p53 function, but actually promote certain aspects of tumor progression (Lang et al., 2004; Olive et al., 2004; Terzian et al., 2008). Elucidation of the molecular mechanisms by which these p53 mutant proteins contribute to tumorigenesis will deepen our understanding of cancer biology as well as potentially offer novel therapeutic targets for mutant p53 expressing tumors.

In this dissertation, we explore the role of mutant p53 proteins and their role in breast tumorigenesis using a three-dimensional culture model system. It is increasingly recognized that the microenvironment that a cell experiences in traditional monolayer culture dramatically differs from that which it experiences *in vivo*. We utilize the 3D ECM model pioneered by the

Bissell and Petersen groups, which allows one to easily distinguish non-malignant and tumorigenic cells based on their morphology and behavior in 3D culture (Barcellos-Hoff et al., 1989; Petersen et al., 1992), to recapitulate physiologic conditions. We identify the mevalonate pathway as upregulated by endogenous p53 mutants and provide evidence that inhibition of this critical cellular pathway may offer a much needed therapeutic target for mutant p53 tumors.

> "Those sterols! Dr. Obisopo frowned…Longbotham had even suggested a connexion fatty alcohols and neoplasms. In other words, cancer might be regarded, in a final analysis, as a symptom of sterol-poisoning."
>
> Aldous Huxley, *After Many a Summer Dies the Swan*, 1939 (Huxley and Rouben Mamoulian Collection (Library of Congress), 1939)

REFERENCES

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V., *et al.* (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell *137*, 87-98.

Ahern, T.P., Pedersen, L., Tarp, M., Cronin-Fenton, D.P., Garne, J.P., Silliman, R.A., Sorensen, H.T., and Lash, T.L. (2011). Statin Prescriptions and Breast Cancer Recurrence Risk: A Danish Nationwide Prospective Cohort Study. J Natl Cancer Inst.

Allred, D.C., Mohsin, S.K., and Fuqua, S.A. (2001). Histological and biological evolution of human premalignant breast disease. Endocr Relat Cancer *8*, 47-61.

Alsner, J., Jensen, V., Kyndi, M., Offersen, B.V., Vu, P., Borresen-Dale, A.L., and Overgaard, J. (2008). A comparison between p53 accumulation determined by immunohistochemistry and TP53 mutations as prognostic variables in tumours from breast cancer patients. Acta Oncol *47*, 600-607.

Aranda, V., Haire, T., Nolan, M.E., Calarco, J.P., Rosenberg, A.Z., Fawcett, J.P., Pawson, T., and Muthuswamy, S.K. (2006). Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. Nat Cell Biol *8*, 1235-1245.

Arias-Romero, L.E., Villamar-Cruz, O., Pacheco, A., Kosoff, R., Huang, M., Muthuswamy, S.K., and Chernoff, J. (2010). A Rac-Pak signaling pathway is essential for ErbB2-mediated transformation of human breast epithelial cancer cells. Oncogene *29*, 5839-5849.

Attardi, L.D., and Jacks, T. (1999). The role of p53 in tumour suppression: lessons from mouse models. Cell Mol Life Sci *55*, 48-63.

Bachelder, R.E., Marchetti, A., Falcioni, R., Soddu, S., and Mercurio, A.M. (1999). Activation of p53 function in carcinoma cells by the alpha6beta4 integrin. J Biol Chem *274*, 20733-20737.

Baigent, C., Keech, A., Kearney, P.M., Blackwell, L., Buck, G., Pollicino, C., Kirby, A., Sourjina, T., Peto, R., Collins, R., *et al.* (2005). Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet *366*, 1267-1278.

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., vanTuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., *et al.* (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science *244*, 217-221.

Baker, S.J., Preisinger, A.C., Jessup, J.M., Paraskeva, C., Markowitz, S., Willson, J.K., Hamilton, S., and Vogelstein, B. (1990). p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Res *50*, 7717-7722.

Barcellos-Hoff, M.H., Aggeler, J., Ram, T.G., and Bissell, M.J. (1989). Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. Development *105*, 223-235.

Bargonetti, J., Chicas, A., White, D., and Prives, C. (1997). p53 represses Sp1 DNA binding and HIV-LTR directed transcription. Cell Mol Biol (Noisy-le-grand) *43*, 935-949.

Barnes, D.E., and Lindahl, T. (2004). Repair and genetic consequences of endogenous DNA base damage in mammalian cells. Annu Rev Genet *38*, 445-476.

Bartek, J., Iggo, R., Gannon, J., and Lane, D.P. (1990). Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene *5*, 893-899.

Basset-Seguin, N., Moles, J.P., Mils, V., Dereure, O., and Guilhou, J.J. (1994). TP53 tumor suppressor gene and skin carcinogenesis. J Invest Dermatol *103*, 102S-106S.

Basso, A.D., Kirschmeier, P., and Bishop, W.R. (2006). Lipid posttranslational modifications. Farnesyl transferase inhibitors. J Lipid Res *47*, 15-31.

Beliveau, A., Mott, J.D., Lo, A., Chen, E.I., Koller, A.A., Yaswen, P., Muschler, J., and Bissell, M.J. (2010). Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. Genes Dev *24*, 2800-2811.

Ben David, Y., Prideaux, V.R., Chow, V., Benchimol, S., and Bernstein, A. (1988). Inactivation of the p53 oncogene by internal deletion or retroviral integration in erythroleukemic cell lines induced by Friend leukemia virus. Oncogene *3*, 179-185.

Bengoechea-Alonso, M.T., and Ericsson, J. (2007). SREBP in signal transduction: cholesterol metabolism and beyond. Curr Opin Cell Biol *19*, 215-222.

Bensaad, K., Le Bras, M., Unsal, K., Strano, S., Blandino, G., Tominaga, O., Rouillard, D., and Soussi, T. (2003). Change of conformation of the DNA-binding domain of p53 is the only key element for binding of and interference with p73. J Biol Chem *278*, 10546-10555.

Bissell, M.J. (1981). The differentiated state of normal and malignant cells or how to define a "normal" cell in culture. Int Rev Cytol *70*, 27-100.

Bissell, M.J. (2007). Architecture Is the Message: The role of extracellular matrix and 3-D structure in tissue-specific gene expression and breast cancer. Pezcoller Found J *16*, 2-17.

Bissell, M.J., Farson, D., and Tung, A.S. (1977). Cell shape and hexose transport in normal and virus-transformed cells in culture. J Supramol Struct *6*, 1-12.

Bissell, M.J., Hall, H.G., and Parry, G. (1982). How does the extracellular matrix direct gene expression? J Theor Biol *99*, 31-68.

Bissell, M.J., Kenny, P.A., and Radisky, D.C. (2005). Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes. Cold Spring Harb Symp Quant Biol *70*, 343-356.

Bissell, M.J., Radisky, D.C., Rizki, A., Weaver, V.M., and Petersen, O.W. (2002). The organizing principle: microenvironmental influences in the normal and malignant breast. Differentiation *70*, 537-546.

Blais, L., Desgagne, A., and LeLorier, J. (2000). 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and the risk of cancer: a nested case-control study. Arch Intern Med *160*, 2363-2368.

Boeckler, F.M., Joerger, A.C., Jaggi, G., Rutherford, T.J., Veprintsev, D.B., and Fersht, A.R. (2008). Targeted rescue of a destabilized mutant of p53 by an in silico screened drug. Proc Natl Acad Sci U S A *105*, 10360-10365.

Borresen-Dale, A.L. (2003). TP53 and breast cancer. Hum Mutat *21*, 292-300.

Bossi, G., Lapi, E., Strano, S., Rinaldo, C., Blandino, G., and Sacchi, A. (2006). Mutant p53 gain of function: reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression. Oncogene *25*, 304-309.

Bossi, G., Marampon, F., Maor-Aloni, R., Zani, B., Rotter, V., Oren, M., Strano, S., Blandino, G., and Sacchi, A. (2008). Conditional RNA interference in vivo to study mutant p53 oncogenic gain of function on tumor malignancy. Cell Cycle *7*, 1870-1879.

Boudreau, N., Sympson, C.J., Werb, Z., and Bissell, M.J. (1995). Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science *267*, 891-893.

Bougeard, G., Sesboue, R., Baert-Desurmont, S., Vasseur, S., Martin, C., Tinat, J., Brugieres, L., Chompret, A., de Paillerets, B.B., Stoppa-Lyonnet, D.*, et al.* (2008). Molecular basis of the Li-Fraumeni syndrome: an update from the French LFS families. J Med Genet *45*, 535-538.

Brazdova, M., Quante, T., Togel, L., Walter, K., Loscher, C., Tichy, V., Cincarova, L., Deppert, W., and Tolstonog, G.V. (2009). Modulation of gene expression in U251 glioblastoma cells by binding of mutant p53 R273H to intronic and intergenic sequences. Nucleic Acids Res *37*, 1486-1500.

Briand, P., Petersen, O.W., and Van Deurs, B. (1987). A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. In Vitro Cell Dev Biol *23*, 181-188.

Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer *9*, 701-713.

Brown, C.J., Cheok, C.F., Verma, C.S., and Lane, D.P. (2011). Reactivation of p53: from peptides to small molecules. Trends Pharmacol Sci *32*, 53-62.

Brown, M.S., and Goldstein, J.L. (1980). Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J Lipid Res *21*, 505-517.

Brown, M.S., and Goldstein, J.L. (2009). Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL. J Lipid Res *50 Suppl*, S15-27.

Browning, D.R., and Martin, R.M. (2007). Statins and risk of cancer: a systematic review and metaanalysis. Int J Cancer *120*, 833-843.

Buchwald, H. (1992). Cholesterol inhibition, cancer, and chemotherapy. Lancet *339*, 1154-1156.

Buganim, Y., Solomon, H., Rais, Y., Kistner, D., Nachmany, I., Brait, M., Madar, S., Goldstein, I., Kalo, E., Adam, N., *et al.* (2010). p53 Regulates the Ras circuit to inhibit the expression of a cancer-related gene signature by various molecular pathways. Cancer Res *70*, 2274-2284.

Bush, J.A., and Li, G. (2002). Cancer chemoresistance: the relationship between p53 and multidrug transporters. Int J Cancer *98*, 323-330.

Buzzai, M., Jones, R.G., Amaravadi, R.K., Lum, J.J., DeBerardinis, R.J., Zhao, F., Viollet, B., and Thompson, C.B. (2007). Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. Cancer Res *67*, 6745-6752.

Bykov, V.J., Issaeva, N., Shilov, A., Hultcrantz, M., Pugacheva, E., Chumakov, P., Bergman, J., Wiman, K.G., and Selivanova, G. (2002). Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. Nat Med *8*, 282-288.

Campbell, M.J., Esserman, L.J., Zhou, Y., Shoemaker, M., Lobo, M., Borman, E., Baehner, F., Kumar, A.S., Adduci, K., Marx, C., *et al.* (2006). Breast cancer growth prevention by statins. Cancer Res *66*, 8707-8714.

Candau, R., Scolnick, D.M., Darpino, P., Ying, C.Y., Halazonetis, T.D., and Berger, S.L. (1997). Two tandem and independent sub-activation domains in the amino terminus of p53 require the adaptor complex for activity. Oncogene *15*, 807-816.

Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.

Cao, Z., Fan-Minogue, H., Bellovin, D.I., Yevtodiyenko, A., Arzeno, J., Yang, Q., Gambhir, S.S., and Felsher, D.W. (2011). MYC Phosphorylation, Activation, and Tumorigenic Potential in Hepatocellular Carcinoma Are Regulated by HMG-CoA Reductase. Cancer Res *71*, 2286-2297.

Caron de Fromentel, C., and Soussi, T. (1992). TP53 tumor suppressor gene: a model for investigating human mutagenesis. Genes Chromosomes Cancer *4*, 1-15.

Carrio, M., Arderiu, G., Myers, C., and Boudreau, N.J. (2005). Homeobox D10 induces phenotypic reversion of breast tumor cells in a three-dimensional culture model. Cancer Res *65*, 7177-7185.

Casey, P.J., Solski, P.A., Der, C.J., and Buss, J.E. (1989). p21ras is modified by a farnesyl isoprenoid. Proc Natl Acad Sci U S A *86*, 8323-8327.

Cauley, J.A., Zmuda, J.M., Lui, L.Y., Hillier, T.A., Ness, R.B., Stone, K.L., Cummings, S.R., and Bauer, D.C. (2003). Lipid-lowering drug use and breast cancer in older women: a prospective study. J Womens Health (Larchmt) *12*, 749-756.

Chang, J., Kim, D.H., Lee, S.W., Choi, K.Y., and Sung, Y.C. (1995). Transactivation ability of p53 transcriptional activation domain is directly related to the binding affinity to TATA-binding protein. J Biol Chem *270*, 25014-25019.

Chen, H.M., Schmeichel, K.L., Mian, I.S., Lelievre, S., Petersen, O.W., and Bissell, M.J. (2000). AZU-1: a candidate breast tumor suppressor and biomarker for tumor progression. Mol Biol Cell *11*, 1357-1367.

Chicas, A., Molina, P., and Bargonetti, J. (2000). Mutant p53 forms a complex with Sp1 on HIV-LTR DNA. Biochem Biophys Res Commun *279*, 383-390.

Chin, K.V., Ueda, K., Pastan, I., and Gottesman, M.M. (1992). Modulation of activity of the promoter of the human MDR1 gene by Ras and p53. Science *255*, 459-462.

Cho, Y., Gorina, S., Jeffrey, P.D., and Pavletich, N.P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science *265*, 346-355.

Christophorou, M.A., Martin-Zanca, D., Soucek, L., Lawlor, E.R., Brown-Swigart, L., Verschuren, E.W., and Evan, G.I. (2005). Temporal dissection of p53 function in vitro and in vivo. Nat Genet *37*, 718-726.

Christophorou, M.A., Ringshausen, I., Finch, A.J., Swigart, L.B., and Evan, G.I. (2006). The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. Nature *443*, 214-217.

Clarke, A.S., Lotz, M.M., Chao, C., and Mercurio, A.M. (1995). Activation of the p21 pathway of growth arrest and apoptosis by the beta 4 integrin cytoplasmic domain. J Biol Chem *270*, 22673-22676.

Clendening, J.W., Pandyra, A., Boutros, P.C., El Ghamrasni, S., Khosravi, F., Trentin, G.A., Martirosyan, A., Hakem, A., Hakem, R., Jurisica, I.*, et al.* (2010). Dysregulation of the mevalonate pathway promotes transformation. Proc Natl Acad Sci U S A *107*, 15051-15056.

Dai, X.M., Ryan, G.R., Hapel, A.J., Dominguez, M.G., Russell, R.G., Kapp, S., Sylvestre, V., and Stanley, E.R. (2002). Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood *99*, 111-120.

Datta, S., and Osborne, T.F. (2005). Activation domains from both monomers contribute to transcriptional stimulation by sterol regulatory element-binding protein dimers. J Biol Chem *280*, 3338-3345.

Davis, T.L., Cress, A.E., Dalkin, B.L., and Nagle, R.B. (2001). Unique expression pattern of the alpha6beta4 integrin and laminin-5 in human prostate carcinoma. Prostate *46*, 240-248.

Davison, T.S., Vagner, C., Kaghad, M., Ayed, A., Caput, D., and Arrowsmith, C.H. (1999). p73 and p63 are homotetramers capable of weak heterotypic interactions with each other but not with p53. J Biol Chem *274*, 18709-18714.

Deb, S., Jackson, C.T., Subler, M.A., and Martin, D.W. (1992). Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. J Virol *66*, 6164-6170.

Debnath, J., and Brugge, J.S. (2005). Modelling glandular epithelial cancers in three-dimensional cultures. Nat Rev Cancer *5*, 675-688.

Debnath, J., Mills, K.R., Collins, N.L., Reginato, M.J., Muthuswamy, S.K., and Brugge, J.S. (2002). The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell *111*, 29-40.

Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003a). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods *30*, 256-268.

Debnath, J., Walker, S.J., and Brugge, J.S. (2003b). Akt activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner. J Cell Biol *163*, 315-326.

DeLeo, A.B., Jay, G., Appella, E., Dubois, G.C., Law, L.W., and Old, L.J. (1979). Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc Natl Acad Sci U S A *76*, 2420-2424.

Dell'Orso, S., Fontemaggi, G., Stambolsky, P., Goeman, F., Voellenkle, C., Levrero, M., Strano, S., Rotter, V., Oren, M., and Blandino, G. (2011). ChIP-on-chip analysis of in vivo mutant p53 binding to selected gene promoters. OMICS *15*, 305-312.

Denoyelle, C., Vasse, M., Korner, M., Mishal, Z., Ganne, F., Vannier, J.P., Soria, J., and Soria, C. (2001). Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits the signaling pathways involved in the invasiveness and metastatic properties of highly invasive breast cancer cell lines: an in vitro study. Carcinogenesis *22*, 1139-1148.

Di Agostino, S., Cortese, G., Monti, O., Dell'Orso, S., Sacchi, A., Eisenstein, M., Citro, G., Strano, S., and Blandino, G. (2008). The disruption of the protein complex mutantp53/p73 increases selectively the response of tumor cells to anticancer drugs. Cell Cycle *7*, 3440-3447.

Di Agostino, S., Strano, S., Emiliozzi, V., Zerbini, V., Mottolese, M., Sacchi, A., Blandino, G., and Piaggio, G. (2006). Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer Cell *10*, 191-202.

Di Como, C.J., Gaiddon, C., and Prives, C. (1999). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. Mol Cell Biol *19*, 1438-1449.

Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A.K., Moore, M., Finlay, C., and Levine, A.J. (1993). Gain of function mutations in p53. Nat Genet *4*, 42-46.

Done, S.J., Eskandarian, S., Bull, S., Redston, M., and Andrulis, I.L. (2001). p53 missense mutations in microdissected high-grade ductal carcinoma in situ of the breast. J Natl Cancer Inst *93*, 700-704.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

Donehower, L.A., and Lozano, G. (2009). 20 years studying p53 functions in genetically engineered mice. Nat Rev Cancer *9*, 831-841.

Dozmorov, M.G., Kyker, K.D., Hauser, P.J., Saban, R., Buethe, D.D., Dozmorov, I., Centola, M.B., Culkin, D.J., and Hurst, R.E. (2008). From microarray to biology: an integrated experimental, statistical and in silico analysis of how the extracellular matrix modulates the phenotype of cancer cells. BMC Bioinformatics *9 Suppl 9*, S4.

Duncan, R.E., El-Sohemy, A., and Archer, M.C. (2004). Mevalonate promotes the growth of tumors derived from human cancer cells in vivo and stimulates proliferation in vitro with enhanced cyclin-dependent kinase-2 activity. J Biol Chem *279*, 33079-33084.

Dutta, U., and Shaw, L.M. (2008). A key tyrosine (Y1494) in the beta4 integrin regulates multiple signaling pathways important for tumor development and progression. Cancer Res *68*, 8779-8787.

Eberle, D., Hegarty, B., Bossard, P., Ferre, P., and Foufelle, F. (2004). SREBP transcription factors: master regulators of lipid homeostasis. Biochimie *86*, 839-848.

Edwards, P.A., Tabor, D., Kast, H.R., and Venkateswaran, A. (2000). Regulation of gene expression by SREBP and SCAP. Biochim Biophys Acta *1529*, 103-113.

el-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. Nat Genet *1*, 45-49.

Eliyahu, D., Raz, A., Gruss, P., Givol, D., and Oren, M. (1984). Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature *312*, 646-649.

Elledge, R.M., Fuqua, S.A., Clark, G.M., Pujol, P., Allred, D.C., and McGuire, W.L. (1993). Prognostic significance of p53 gene alterations in node-negative breast cancer. Breast Cancer Res Treat *26*, 225-235.

Elsdale, T., and Bard, J. (1972). Collagen substrata for studies on cell behavior. J Cell Biol *54*, 626-637.

Emerman, J.T., Enami, J., Pitelka, D.R., and Nandi, S. (1977). Hormonal effects on intracellular and secreted casein in cultures of mouse mammary epithelial cells on floating collagen membranes. Proc Natl Acad Sci U S A *74*, 4466-4470.

Fears, R. (1981). The contribution of the cholesterol biosynthetic pathway to intermediary metabolism and cell function. Biochem J *199*, 1-7.

Ferenczy, A., and Franco, E. (2002). Persistent human papillomavirus infection and cervical neoplasia. Lancet Oncol *3*, 11-16.

Finlay, C.A., Hinds, P.W., and Levine, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. Cell *57*, 1083-1093.

Flores, E.R., Sengupta, S., Miller, J.B., Newman, J.J., Bronson, R., Crowley, D., Yang, A., McKeon, F., and Jacks, T. (2005). Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. Cancer Cell *7*, 363-373.

Fontemaggi, G., Dell'Orso, S., Trisciuoglio, D., Shay, T., Melucci, E., Fazi, F., Terrenato, I., Mottolese, M., Muti, P., Domany, E.*, et al.* (2009). The execution of the transcriptional axis mutant p53, E2F1 and ID4 promotes tumor neo-angiogenesis. Nat Struct Mol Biol *16*, 1086-1093.

Foster, B.A., Coffey, H.A., Morin, M.J., and Rastinejad, F. (1999). Pharmacological rescue of mutant p53 conformation and function. Science *286*, 2507-2510.

Fournier, M.V., Fata, J.E., Martin, K.J., Yaswen, P., and Bissell, M.J. (2009). Interaction of E-cadherin and PTEN regulates morphogenesis and growth arrest in human mammary epithelial cells. Cancer Res *69*, 4545-4552.

Fournier, M.V., and Martin, K.J. (2006). Transcriptome profiling in clinical breast cancer: from 3D culture models to prognostic signatures. J Cell Physiol *209*, 625-630.

Fournier, M.V., Martin, K.J., Kenny, P.A., Xhaja, K., Bosch, I., Yaswen, P., and Bissell, M.J. (2006). Gene expression signature in organized and growth-arrested mammary acini predicts good outcome in breast cancer. Cancer Res *66*, 7095-7102.

Frazier, M.W., He, X., Wang, J., Gu, Z., Cleveland, J.L., and Zambetti, G.P. (1998). Activation of c-myc gene expression by tumor-derived p53 mutants requires a discrete C-terminal domain. Mol Cell Biol *18*, 3735-3743.

Fried, L.F. (2008). Effects of HMG-CoA reductase inhibitors (statins) on progression of kidney disease. Kidney Int *74*, 571-576.

Funk, W.D., Pak, D.T., Karas, R.H., Wright, W.E., and Shay, J.W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. Mol Cell Biol *12*, 2866-2871.

Furuta, S., Jiang, X., Gu, B., Cheng, E., Chen, P.L., and Lee, W.H. (2005). Depletion of BRCA1 impairs differentiation but enhances proliferation of mammary epithelial cells. Proc Natl Acad Sci U S A *102*, 9176-9181.

Gabarra, V., Cho, S., Ramirez, M., Ren, Y., Chen, L.L., Cheung, A., Cao, X., Rennard, R., Unruh, K.R., Graff, C.P., *et al.* (2010). Antibodies directed to alpha6beta4 highlight the adhesive and signaling functions of the integrin in breast cancer cell lines. Cancer Biol Ther *9*, 437-445.

Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. (2001). A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol Cell Biol *21*, 1874-1887.

Garwood, E.R., Kumar, A.S., Baehner, F.L., Moore, D.H., Au, A., Hylton, N., Flowers, C.I., Garber, J., Lesnikoski, B.A., Hwang, E.S., *et al.* (2010). Fluvastatin reduces proliferation and increases apoptosis in women with high grade breast cancer. Breast Cancer Res Treat *119*, 137-144.

Gilcrease, M.Z. (2007). Integrin signaling in epithelial cells. Cancer Lett *247*, 1-25.

Girardini, J.E., Napoli, M., Piazza, S., Rustighi, A., Marotta, C., Radaelli, E., Capaci, V., Jordan, L., Quinlan, P., Thompson, A., *et al.* (2011). A Pin1/mutant p53 axis promotes aggressiveness in breast cancer. Cancer Cell *20*, 79-91.

Gohler, T., Jager, S., Warnecke, G., Yasuda, H., Kim, E., and Deppert, W. (2005). Mutant p53 proteins bind DNA in a DNA structure-selective mode. Nucleic Acids Res *33*, 1087-1100.

Goldstein, I., Marcel, V., Olivier, M., Oren, M., Rotter, V., and Hainaut, P. (2011). Understanding wild-type and mutant p53 activities in human cancer: new landmarks on the way to targeted therapies. Cancer Gene Ther *18*, 2-11.

Goldstein, J.L., and Brown, M.S. (1990). Regulation of the mevalonate pathway. Nature *343*, 425-430.

Goldstein, J.L., DeBose-Boyd, R.A., and Brown, M.S. (2006). Protein sensors for membrane sterols. Cell *124*, 35-46.

Graaf, M.R., Richel, D.J., van Noorden, C.J., and Guchelaar, H.J. (2004). Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer. Cancer Treat Rev *30*, 609-641.

Guo, W., and Giancotti, F.G. (2004). Integrin signalling during tumour progression. Nat Rev Mol Cell Biol *5*, 816-826.

Guo, W., Pylayeva, Y., Pepe, A., Yoshioka, T., Muller, W.J., Inghirami, G., and Giancotti, F.G. (2006). Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. Cell *126*, 489-502.

Gurtner, A., Starace, G., Norelli, G., Piaggio, G., Sacchi, A., and Bossi, G. (2010). Mutant p53-induced up-regulation of mitogen-activated protein kinase kinase 3 contributes to gain of function. J Biol Chem *285*, 14160-14169.

Gymnopoulos, M., Elsliger, M.A., and Vogt, P.K. (2007). Rare cancer-specific mutations in PIK3CA show gain of function. Proc Natl Acad Sci U S A *104*, 5569-5574.

Hall, H.G., Farson, D.A., and Bissell, M.J. (1982). Lumen formation by epithelial cell lines in response to collagen overlay: a morphogenetic model in culture. Proc Natl Acad Sci U S A *79*, 4672-4676.

Hansen, R.K., and Bissell, M.J. (2000). Tissue architecture and breast cancer: the role of extracellular matrix and steroid hormones. Endocr Relat Cancer *7*, 95-113.

Harris, C.C., and Hollstein, M. (1993). Clinical implications of the p53 tumor-suppressor gene. N Engl J Med *329*, 1318-1327.

Haupt, S., di Agostino, S., Mizrahi, I., Alsheich-Bartok, O., Voorhoeve, M., Damalas, A., Blandino, G., and Haupt, Y. (2009). Promyelocytic leukemia protein is required for gain of function by mutant p53. Cancer Res *69*, 4818-4826.

Hay, E.D., and Dodson, J.W. (1973). Secretion of collagen by corneal epithelium. I. Morphology of the collagenous products produced by isolated epithelia grown on frozen-killed lens. J Cell Biol *57*, 190-213.

He, Q., Huang, B., Zhao, J., Zhang, Y., Zhang, S., and Miao, J. (2008). Knockdown of integrin beta4-induced autophagic cell death associated with P53 in A549 lung adenocarcinoma cells. FEBS J *275*, 5725-5732.

Hebner, C., Weaver, V.M., and Debnath, J. (2008). Modeling morphogenesis and oncogenesis in three-dimensional breast epithelial cultures. Annu Rev Pathol *3*, 313-339.

Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D.D., and Kirn, D.H. (1997). ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. Nat Med *3*, 639-645.

Herr, R., Wohrle, F.U., Danke, C., Berens, C., and Brummer, T. (2011). A novel MCF-10A line allowing conditional oncogene expression in 3D culture. Cell Commun Signal *9*, 17.

Hinds, P., Finlay, C., and Levine, A.J. (1989). Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J Virol *63*, 739-746.

Hinds, P.W., Finlay, C.A., Quartin, R.S., Baker, S.J., Fearon, E.R., Vogelstein, B., and Levine, A.J. (1990). Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes. Cell Growth Differ *1*, 571-580.

Ho, G.H., Calvano, J.E., Bisogna, M., Borgen, P.I., Rosen, P.P., Tan, L.K., and Van Zee, K.J. (2000). In microdissected ductal carcinoma in situ, HER-2/neu amplification, but not p53 mutation, is associated with high nuclear grade and comedo histology. Cancer *89*, 2153-2160.

Hu, W., Zhang, C., Wu, R., Sun, Y., Levine, A., and Feng, Z. (2010). Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. Proc Natl Acad Sci U S A *107*, 7455-7460.

Hua, X., Yokoyama, C., Wu, J., Briggs, M.R., Brown, M.S., Goldstein, J.L., and Wang, X. (1993). SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. Proc Natl Acad Sci U S A *90*, 11603-11607.

Huang, J., Hardy, J.D., Sun, Y., and Shively, J.E. (1999). Essential role of biliary glycoprotein (CD66a) in morphogenesis of the human mammary epithelial cell line MCF10F. J Cell Sci *112 (Pt 23)*, 4193-4205.

Humphreys, R.C., Krajewska, M., Krnacik, S., Jaeger, R., Weiher, H., Krajewski, S., Reed, J.C., and Rosen, J.M. (1996). Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. Development *122*, 4013-4022.

Huxley, A., and Rouben Mamoulian Collection (Library of Congress) (1939). After many a summer dies the swan (New York, London,, Harper & brothers).

Imbriano, C., Gurtner, A., Cocchiarella, F., Di Agostino, S., Basile, V., Gostissa, M., Dobbelstein, M., Del Sal, G., Piaggio, G., and Mantovani, R. (2005). Direct p53 transcriptional repression: in vivo analysis of CCAAT-containing G2/M promoters. Mol Cell Biol *25*, 3737-3751.

Irie, H.Y., Pearline, R.V., Grueneberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S., and Brugge, J.S. (2005). Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J Cell Biol *171*, 1023-1034.

Irie, H.Y., Shrestha, Y., Selfors, L.M., Frye, F., Iida, N., Wang, Z., Zou, L., Yao, J., Lu, Y., Epstein, C.B., *et al.* (2010). PTK6 regulates IGF-1-induced anchorage-independent survival. PLoS One *5*, e11729.

Irwin, M.S., and Kaelin, W.G., Jr. (2001). Role of the newer p53 family proteins in malignancy. Apoptosis *6*, 17-29.

Irwin, M.S., Kondo, K., Marin, M.C., Cheng, L.S., Hahn, W.C., and Kaelin, W.G., Jr. (2003). Chemosensitivity linked to p73 function. Cancer Cell *3*, 403-410.

Isakoff, S.J., Engelman, J.A., Irie, H.Y., Luo, J., Brachmann, S.M., Pearline, R.V., Cantley, L.C., and Brugge, J.S. (2005). Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. Cancer Res *65*, 10992-11000.

Issaeva, N., Bozko, P., Enge, M., Protopopova, M., Verhoef, L.G., Masucci, M., Pramanik, A., and Selivanova, G. (2004). Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat Med *10*, 1321-1328.

Itoh, M., Nelson, C.M., Myers, C.A., and Bissell, M.J. (2007). Rap1 integrates tissue polarity, lumen formation, and tumorigenic potential in human breast epithelial cells. Cancer Res *67*, 4759-4766.

Iwanaga, Y., and Jeang, K.T. (2002). Expression of mitotic spindle checkpoint protein hsMAD1 correlates with cellular proliferation and is activated by a gain-of-function p53 mutant. Cancer Res *62*, 2618-2624.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., and Thun, M.J. (2009). Cancer statistics, 2009. CA Cancer J Clin *59*, 225-249.

Jung, K.K., Liu, X.W., Chirco, R., Fridman, R., and Kim, H.R. (2006). Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein. EMBO J *25*, 3934-3942.

Kacinski, B.M., Scata, K.A., Carter, D., Yee, L.D., Sapi, E., King, B.L., Chambers, S.K., Jones, M.A., Pirro, M.H., Stanley, E.R., *et al.* (1991). FMS (CSF-1 receptor) and CSF-1 transcripts and protein are expressed by human breast carcinomas in vivo and in vitro. Oncogene *6*, 941-952.

Kamisuki, S., Mao, Q., Abu-Elheiga, L., Gu, Z., Kugimiya, A., Kwon, Y., Shinohara, T., Kawazoe, Y., Sato, S., Asakura, K., *et al.* (2009). A small molecule that blocks fat synthesis by inhibiting the activation of SREBP. Chem Biol *16*, 882-892.

Katz, M.S., Minsky, B.D., Saltz, L.B., Riedel, E., Chessin, D.B., and Guillem, J.G. (2005). Association of statin use with a pathologic complete response to neoadjuvant chemoradiation for rectal cancer. Int J Radiat Oncol Biol Phys *62*, 1363-1370.

Kenny, P.A., and Bissell, M.J. (2007). Targeting TACE-dependent EGFR ligand shedding in breast cancer. J Clin Invest *117*, 337-345.

Kenny, P.A., Lee, G.Y., Myers, C.A., Neve, R.M., Semeiks, J.R., Spellman, P.T., Lorenz, K., Lee, E.H., Barcellos-Hoff, M.H., Petersen, O.W., *et al.* (2007). The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol *1*, 84-96.

Kenzelmann Broz, D., and Attardi, L.D. (2010). In vivo analysis of p53 tumor suppressor function using genetically engineered mouse models. Carcinogenesis *31*, 1311-1318.

Kessenbrock, K., Plaks, V., and Werb, Z. (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. Cell *141*, 52-67.

Khurana, V., Bejjanki, H.R., Caldito, G., and Owens, M.W. (2007a). Statins reduce the risk of lung cancer in humans: a large case-control study of US veterans. Chest *131*, 1282-1288.

Khurana, V., Sheth, A., Caldito, G., and Barkin, J.S. (2007b). Statins reduce the risk of pancreatic cancer in humans: a case-control study of half a million veterans. Pancreas *34*, 260-265.

Kidera, Y., Tsubaki, M., Yamazoe, Y., Shoji, K., Nakamura, H., Ogaki, M., Satou, T., Itoh, T., Isozaki, M., Kaneko, J., *et al.* (2010). Reduction of lung metastasis, cell invasion, and adhesion in mouse melanoma by statin-induced blockade of the Rho/Rho-associated coiled-coil-containing protein kinase pathway. J Exp Clin Cancer Res *29*, 127.

Kim, E., and Deppert, W. (2003). The complex interactions of p53 with target DNA: we learn as we go. Biochem Cell Biol *81*, 141-150.

Kim, E., and Deppert, W. (2004). Transcriptional activities of mutant p53: when mutations are more than a loss. J Cell Biochem *93*, 878-886.

Kim, E., and Deppert, W. (2007). Interactions of mutant p53 with DNA: guilt by association. Oncogene *26*, 2185-2190.

Kleinman, H.K., McGarvey, M.L., Hassell, J.R., Star, V.L., Cannon, F.B., Laurie, G.W., and Martin, G.R. (1986). Basement membrane complexes with biological activity. Biochemistry *25*, 312-318.

Koga, H., and Deppert, W. (2000). Identification of genomic DNA sequences bound by mutant p53 protein (Gly245-->Ser) in vivo. Oncogene *19*, 4178-4183.

Koyuturk, M., Ersoz, M., and Altiok, N. (2007). Simvastatin induces apoptosis in human breast cancer cells: p53 and estrogen receptor independent pathway requiring signalling through JNK. Cancer Lett *250*, 220-228.

Kravchenko, J.E., Ilyinskaya, G.V., Komarov, P.G., Agapova, L.S., Kochetkov, D.V., Strom, E., Frolova, E.I., Kovriga, I., Gudkov, A.V., Feinstein, E., *et al.* (2008). Small-molecule RETRA suppresses mutant p53-bearing cancer cells through a p73-dependent salvage pathway. Proc Natl Acad Sci U S A *105*, 6302-6307.

Kumar, A.S., Benz, C.C., Shim, V., Minami, C.A., Moore, D.H., and Esserman, L.J. (2008). Estrogen receptor-negative breast cancer is less likely to arise among lipophilic statin users. Cancer Epidemiol Biomarkers Prev *17*, 1028-1033.

Kumar, A.S., Campbell, M., Benz, C.C., and Esserman, L.J. (2006). A call for clinical trials: lipophilic statins may prove effective in treatment and prevention of particular breast cancer subtypes. J Clin Oncol *24*, 2127; author reply 2127-2128.

Kussie, P.H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A.J., and Pavletich, N.P. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science *274*, 948-953.

Kwan, M.L., Habel, L.A., Flick, E.D., Quesenberry, C.P., and Caan, B. (2008). Post-diagnosis statin use and breast cancer recurrence in a prospective cohort study of early stage breast cancer survivors. Breast Cancer Res Treat *109*, 573-579.

Lane, D., and Levine, A. (2010). p53 Research: the past thirty years and the next thirty years. Cold Spring Harb Perspect Biol *2*, a000893.

Lane, D.P., and Crawford, L.V. (1979). T antigen is bound to a host protein in SV40-transformed cells. Nature *278*, 261-263.

Lang, G.A., Iwakuma, T., Suh, Y.A., Liu, G., Rao, V.A., Parant, J.M., Valentin-Vega, Y.A., Terzian, T., Caldwell, L.C., Strong, L.C., *et al.* (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell *119*, 861-872.

Langan, T.J., and Volpe, J.J. (1986). Obligatory relationship between the sterol biosynthetic pathway and DNA synthesis and cellular proliferation in glial primary cultures. J Neurochem *46*, 1283-1291.

Langerod, A., Zhao, H., Borgan, O., Nesland, J.M., Bukholm, I.R., Ikdahl, T., Karesen, R., Borresen-Dale, A.L., and Jeffrey, S.S. (2007). TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. Breast Cancer Res *9*, R30.

Laptenko, O., and Prives, C. (2006). Transcriptional regulation by p53: one protein, many possibilities. Cell Death Differ *13*, 951-961.

Larsson, O. (1996). HMG-CoA reductase inhibitors: role in normal and malignant cells. Crit Rev Oncol Hematol *22*, 197-212.

Lasky, T., and Silbergeld, E. (1996). P53 mutations associated with breast, colorectal, liver, lung, and ovarian cancers. Environ Health Perspect *104*, 1324-1331.

Lavra, L., Ulivieri, A., Rinaldo, C., Dominici, R., Volante, M., Luciani, E., Bartolazzi, A., Frasca, F., Soddu, S., and Sciacchitano, S. (2009). Gal-3 is stimulated by gain-of-function p53 mutations and modulates chemoresistance in anaplastic thyroid carcinomas. J Pathol *218*, 66-75.

Lee, E.Y., Parry, G., and Bissell, M.J. (1984). Modulation of secreted proteins of mouse mammary epithelial cells by the collagenous substrata. J Cell Biol *98*, 146-155.

Lee, Y.I., Lee, S., Das, G.C., Park, U.S., and Park, S.M. (2000). Activation of the insulin-like growth factor II transcription by aflatoxin B1 induced p53 mutant 249 is caused by activation of transcription complexes; implications for a gain-of-function during the formation of hepatocellular carcinoma. Oncogene *19*, 3717-3726.

Levine, A.J., and Oren, M. (2009). The first 30 years of p53: growing ever more complex. Nat Rev Cancer *9*, 749-758.

Levine, A.J., Wu, M.C., Chang, A., Silver, A., Attiyeh, E.F., Lin, J., and Epstein, C.B. (1995). The spectrum of mutations at the p53 locus. Evidence for tissue-specific mutagenesis, selection of mutant alleles, and a "gain of function" phenotype. Ann N Y Acad Sci *768*, 111-128.

Li, D., Marchenko, N.D., and Moll, U.M. (2011a). SAHA shows preferential cytotoxicity in mutant p53 cancer cells by destabilizing mutant p53 through inhibition of the HDAC6-Hsp90 chaperone axis. Cell Death Differ.

Li, D., Marchenko, N.D., Schulz, R., Fischer, V., Velasco-Hernandez, T., Talos, F., and Moll, U.M. (2011b). Functional inactivation of endogenous MDM2 and CHIP by HSP90 causes aberrant stabilization of mutant p53 in human cancer cells. Mol Cancer Res *9*, 577-588.

Li, F.P., and Fraumeni, J.F., Jr. (1969a). Rhabdomyosarcoma in children: epidemiologic study and identification of a familial cancer syndrome. J Natl Cancer Inst *43*, 1365-1373.

Li, F.P., and Fraumeni, J.F., Jr. (1969b). Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome? Ann Intern Med *71*, 747-752.

Li, Y., and Prives, C. (2007). Are interactions with p63 and p73 involved in mutant p53 gain of oncogenic function? Oncogene *26*, 2220-2225.

Li, Y., Xu, S., Mihaylova, M.M., Zheng, B., Hou, X., Jiang, B., Park, O., Luo, Z., Lefai, E., Shyy, J.Y., *et al.* (2011c). AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. Cell Metab *13*, 376-388.

Lim, L.Y., Vidnovic, N., Ellisen, L.W., and Leong, C.O. (2009). Mutant p53 mediates survival of breast cancer cells. Br J Cancer *101*, 1606-1612.

Lin, J., Chen, J., Elenbaas, B., and Levine, A.J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes Dev *8*, 1235-1246.

Lin, J., Teresky, A.K., and Levine, A.J. (1995). Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants. Oncogene *10*, 2387-2390.

Linzer, D.I., and Levine, A.J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell *17*, 43-52.

Lipscomb, E.A., Simpson, K.J., Lyle, S.R., Ring, J.E., Dugan, A.S., and Mercurio, A.M. (2005). The alpha6beta4 integrin maintains the survival of human breast carcinoma cells in vivo. Cancer Res *65*, 10970-10976.

Litzenburger, B.C., Kim, H.J., Kuiatse, I., Carboni, J.M., Attar, R.M., Gottardis, M.M., Fairchild, C.R., and Lee, A.V. (2009). BMS-536924 reverses IGF-IR-induced transformation of mammary

epithelial cells and causes growth inhibition and polarization of MCF7 cells. Clin Cancer Res *15*, 226-237.

Liu, G., McDonnell, T.J., Montes de Oca Luna, R., Kapoor, M., Mims, B., El-Naggar, A.K., and Lozano, G. (2000). High metastatic potential in mice inheriting a targeted p53 missense mutation. Proc Natl Acad Sci U S A *97*, 4174-4179.

Liu, G., Parant, J.M., Lang, G., Chau, P., Chavez-Reyes, A., El-Naggar, A.K., Multani, A., Chang, S., and Lozano, G. (2004a). Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nat Genet *36*, 63-68.

Liu, H., Radisky, D.C., Wang, F., and Bissell, M.J. (2004b). Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. J Cell Biol *164*, 603-612.

Liu, K., Ling, S., and Lin, W.C. (2011). TopBP1 mediates mutant p53 gain of function through NF-Y and p63/p73. Mol Cell Biol.

Liu, X.W., Taube, M.E., Jung, K.K., Dong, Z., Lee, Y.J., Roshy, S., Sloane, B.F., Fridman, R., and Kim, H.R. (2005). Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells from extrinsic cell death: a potential oncogenic activity of tissue inhibitor of metalloproteinase-1. Cancer Res *65*, 898-906.

Lochter, A., Galosy, S., Muschler, J., Freedman, N., Werb, Z., and Bissell, M.J. (1997). Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. J Cell Biol *139*, 1861-1872.

Lu, S., Simin, K., Khan, A., and Mercurio, A.M. (2008). Analysis of integrin beta4 expression in human breast cancer: association with basal-like tumors and prognostic significance. Clin Cancer Res *14*, 1050-1058.

Ludes-Meyers, J.H., Subler, M.A., Shivakumar, C.V., Munoz, R.M., Jiang, P., Bigger, J.E., Brown, D.R., Deb, S.P., and Deb, S. (1996). Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol Cell Biol *16*, 6009-6019.

Luo, J.L., Yang, Q., Tong, W.M., Hergenhahn, M., Wang, Z.Q., and Hollstein, M. (2001). Knock-in mice with a chimeric human/murine p53 gene develop normally and show wild-type p53 responses to DNA damaging agents: a new biomedical research tool. Oncogene *20*, 320-328.

Maddocks, O.D., and Vousden, K.H. (2011). Metabolic regulation by p53. J Mol Med (Berl) *89*, 237-245.

Mailleux, A.A., Overholtzer, M., Schmelzle, T., Bouillet, P., Strasser, A., and Brugge, J.S. (2007). BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. Dev Cell *12*, 221-234.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Jr., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A., *et al.* (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science *250*, 1233-1238.

Marin, M.C., Jost, C.A., Brooks, L.A., Irwin, M.S., O'Nions, J., Tidy, J.A., James, N., McGregor, J.M., Harwood, C.A., Yulug, I.G., *et al.* (2000). A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. Nat Genet *25*, 47-54.

Marine, J.C., Francoz, S., Maetens, M., Wahl, G., Toledo, F., and Lozano, G. (2006). Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. Cell Death Differ *13*, 927-934.

Martin, K.J., Patrick, D.R., Bissell, M.J., and Fournier, M.V. (2008). Prognostic breast cancer signature identified from 3D culture model accurately predicts clinical outcome across independent datasets. PLoS One *3*, e2994.

Matas, D., Sigal, A., Stambolsky, P., Milyavsky, M., Weisz, L., Schwartz, D., Goldfinger, N., and Rotter, V. (2001). Integrity of the N-terminal transcription domain of p53 is required for mutant p53 interference with drug-induced apoptosis. EMBO J *20*, 4163-4172.

Maurer-Stroh, S., Koranda, M., Benetka, W., Schneider, G., Sirota, F.L., and Eisenhaber, F. (2007). Towards complete sets of farnesylated and geranylgeranylated proteins. PLoS Comput Biol *3*, e66.

McPherson, R., and Gauthier, A. (2004). Molecular regulation of SREBP function: the Insig-SCAP connection and isoform-specific modulation of lipid synthesis. Biochem Cell Biol *82*, 201-211.

Meiners, S., Brinkmann, V., Naundorf, H., and Birchmeier, W. (1998). Role of morphogenetic factors in metastasis of mammary carcinoma cells. Oncogene *16*, 9-20.

Michalopoulos, G., and Pitot, H.C. (1975). Primary culture of parenchymal liver cells on collagen membranes. Morphological and biochemical observations. Exp Cell Res *94*, 70-78.

Mizuno, H., Spike, B.T., Wahl, G.M., and Levine, A.J. (2010). Inactivation of p53 in breast cancers correlates with stem cell transcriptional signatures. Proc Natl Acad Sci U S A *107*, 22745-22750.

Mo, H., and Elson, C.E. (2004). Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. Exp Biol Med (Maywood) *229*, 567-585.

Muller, B.F., Paulsen, D., and Deppert, W. (1996). Specific binding of MAR/SAR DNA-elements by mutant p53. Oncogene *12*, 1941-1952.

Muller, P.A., Caswell, P.T., Doyle, B., Iwanicki, M.P., Tan, E.H., Karim, S., Lukashchuk, N., Gillespie, D.A., Ludwig, R.L., Gosselin, P., *et al.* (2009). Mutant p53 drives invasion by promoting integrin recycling. Cell *139*, 1327-1341.

Muschler, J., Levy, D., Boudreau, R., Henry, M., Campbell, K., and Bissell, M.J. (2002). A role for dystroglycan in epithelial polarization: loss of function in breast tumor cells. Cancer Res *62*, 7102-7109.

Muthuswamy, S.K., Li, D., Lelievre, S., Bissell, M.J., and Brugge, J.S. (2001). ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol *3*, 785-792.

Niemann, C., Brinkmann, V., Spitzer, E., Hartmann, G., Sachs, M., Naundorf, H., and Birchmeier, W. (1998). Reconstitution of mammary gland development in vitro: requirement of c-met and c-erbB2 signaling for branching and alveolar morphogenesis. J Cell Biol *143*, 533-545.

Nikolopoulos, S.N., Blaikie, P., Yoshioka, T., Guo, W., and Giancotti, F.G. (2004). Integrin beta4 signaling promotes tumor angiogenesis. Cancer Cell *6*, 471-483.

Nilsson, S., Huelsenbeck, J., and Fritz, G. (2011). Mevalonate pathway inhibitors affect anticancer drug-induced cell death and DNA damage response of human sarcoma cells. Cancer Lett *304*, 60-69.

Ojakian, G.K., Nelson, W.J., and Beck, K.A. (1997). Mechanisms for de novo biogenesis of an apical membrane compartment in groups of simple epithelial cells surrounded by extracellular matrix. J Cell Sci *110 (Pt 22)*, 2781-2794.

Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell *119*, 847-860.

Olivier, M., Langerod, A., Carrieri, P., Bergh, J., Klaar, S., Eyfjord, J., Theillet, C., Rodriguez, C., Lidereau, R., Bieche, I., *et al.* (2006). The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. Clin Cancer Res *12*, 1157-1167.

Oren, M., and Rotter, V. (2010). Mutant p53 gain-of-function in cancer. Cold Spring Harb Perspect Biol *2*, a001107.

Osborne, T.F. (2000). Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. J Biol Chem *275*, 32379-32382.

Osborne, T.F., and Espenshade, P.J. (2009). Evolutionary conservation and adaptation in the mechanism that regulates SREBP action: what a long, strange tRIP it's been. Genes Dev *23*, 2578-2591.

Overholtzer, M., Zhang, J., Smolen, G.A., Muir, B., Li, W., Sgroi, D.C., Deng, C.X., Brugge, J.S., and Haber, D.A. (2006). Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. Proc Natl Acad Sci U S A *103*, 12405-12410.

Parada, L.F., Land, H., Weinberg, R.A., Wolf, D., and Rotter, V. (1984). Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. Nature *312*, 649-651.

Peart, M.J., and Prives, C. (2006). Mutant p53 gain of function: the NF-Y connection. Cancer Cell *10*, 173-174.

Pedersen, T.R., Wilhelmsen, L., Faergeman, O., Strandberg, T.E., Thorgeirsson, G., Troedsson, L., Kristianson, J., Berg, K., Cook, T.J., Haghfelt, T.*, et al.* (2000). Follow-up study of patients randomized in the Scandinavian simvastatin survival study (4S) of cholesterol lowering. Am J Cardiol *86*, 257-262.

Peng, Y., Chen, L., Li, C., Lu, W., and Chen, J. (2001). Inhibition of MDM2 by hsp90 contributes to mutant p53 stabilization. Journal of Biological Chemistry *276*, 40583-40590.

Petersen, O.W., Ronnov-Jessen, L., Howlett, A.R., and Bissell, M.J. (1992). Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc Natl Acad Sci U S A *89*, 9064-9068.

Petitjean, A., Achatz, M.I., Borresen-Dale, A.L., Hainaut, P., and Olivier, M. (2007a). TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene *26*, 2157-2165.

Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S.V., Hainaut, P., and Olivier, M. (2007b). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat *28*, 622-629.

Pietsch, E.C., Sykes, S.M., McMahon, S.B., and Murphy, M.E. (2008). The p53 family and programmed cell death. Oncogene *27*, 6507-6521.

Polager, S., and Ginsberg, D. (2009). p53 and E2f: partners in life and death. Nat Rev Cancer *9*, 738-748.

Poynter, J.N., Gruber, S.B., Higgins, P.D., Almog, R., Bonner, J.D., Rennert, H.S., Low, M., Greenson, J.K., and Rennert, G. (2005). Statins and the risk of colorectal cancer. N Engl J Med *352*, 2184-2192.

Poyurovsky, M.V., and Prives, C. (2006). Unleashing the power of p53: lessons from mice and men. Genes Dev *20*, 125-131.

Preuss, U., Kreutzfeld, R., and Scheidtmann, K.H. (2000). Tumor-derived p53 mutant C174Y is a gain-of-function mutant which activates the fos promoter and enhances colony formation. Int J Cancer *88*, 162-171.

Prives, C., and Hall, P.A. (1999). The p53 pathway. J Pathol *187*, 112-126.

Prives, C., and White, E. (2008). Does control of mutant p53 by Mdm2 complicate cancer therapy? Genes Dev *22*, 1259-1264.

Prowell, T.M., Stearns, V., and Trock, B. (2006). Lipophilic statins merit additional study for breast cancer chemoprevention. J Clin Oncol *24*, 2128-2129; author reply 2129.

Pugacheva, E.N., Ivanov, A.V., Kravchenko, J.E., Kopnin, B.P., Levine, A.J., and Chumakov, P.M. (2002). Novel gain of function activity of p53 mutants: activation of the dUTPase gene expression leading to resistance to 5-fluorouracil. Oncogene *21*, 4595-4600.

Quesney-Huneeus, V., Wiley, M.H., and Siperstein, M.D. (1979). Essential role for mevalonate synthesis in DNA replication. Proc Natl Acad Sci U S A *76*, 5056-5060.

Radisky, D.C., Levy, D.D., Littlepage, L.E., Liu, H., Nelson, C.M., Fata, J.E., Leake, D., Godden, E.L., Albertson, D.G., Nieto, M.A.*, et al.* (2005). Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature *436*, 123-127.

Reed, B.D., Charos, A.E., Szekely, A.M., Weissman, S.M., and Snyder, M. (2008). Genome-wide occupancy of SREBP1 and its partners NFY and SP1 reveals novel functional roles and combinatorial regulation of distinct classes of genes. PLoS Genet *4*, e1000133.

Reginato, M.J., Mills, K.R., Becker, E.B., Lynch, D.K., Bonni, A., Muthuswamy, S.K., and Brugge, J.S. (2005). Bim regulation of lumen formation in cultured mammary epithelial acini is targeted by oncogenes. Mol Cell Biol *25*, 4591-4601.

Reginato, M.J., Mills, K.R., Paulus, J.K., Lynch, D.K., Sgroi, D.C., Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003). Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat Cell Biol *5*, 733-740.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol *9*, 402-412.

Rippin, T.M., Bykov, V.J., Freund, S.M., Selivanova, G., Wiman, K.G., and Fersht, A.R. (2002). Characterization of the p53-rescue drug CP-31398 in vitro and in living cells. Oncogene *21*, 2119-2129.

Rodriguez-Cruz, M., Tovar, A.R., Palacios-Gonzalez, B., Del Prado, M., and Torres, N. (2006). Synthesis of long-chain polyunsaturated fatty acids in lactating mammary gland: role of Delta5 and Delta6 desaturases, SREBP-1, PPARalpha, and PGC-1. J Lipid Res *47*, 553-560.

Roskelley, C.D., Desprez, P.Y., and Bissell, M.J. (1994). Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. Proc Natl Acad Sci U S A *91*, 12378-12382.

Rotter, V. (1983). p53, a transformation-related cellular-encoded protein, can be used as a biochemical marker for the detection of primary mouse tumor cells. Proc Natl Acad Sci U S A *80*, 2613-2617.

Rowan, S., Ludwig, R.L., Haupt, Y., Bates, S., Lu, X., Oren, M., and Vousden, K.H. (1996). Specific loss of apoptotic but not cell-cycle arrest function in a human tumor derived p53 mutant. EMBO J *15*, 827-838.

Rudolph, M.C., McManaman, J.L., Phang, T., Russell, T., Kominsky, D.J., Serkova, N.J., Stein, T., Anderson, S.M., and Neville, M.C. (2007). Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine. Physiol Genomics *28*, 323-336.

Sampath, J., Sun, D., Kidd, V.J., Grenet, J., Gandhi, A., Shapiro, L.H., Wang, Q., Zambetti, G.P., and Schuetz, J.D. (2001). Mutant p53 cooperates with ETS and selectively up-regulates human MDR1 not MRP1. J Biol Chem *276*, 39359-39367.

Sanchez, C.A., Rodriguez, E., Varela, E., Zapata, E., Paez, A., Masso, F.A., Montano, L.F., and Loopez-Marure, R. (2008). Statin-induced inhibition of MCF-7 breast cancer cell proliferation is related to cell cycle arrest and apoptotic and necrotic cell death mediated by an enhanced oxidative stress. Cancer Invest *26*, 698-707.

Sandal, T., Valyi-Nagy, K., Spencer, V.A., Folberg, R., Bissell, M.J., and Maniotis, A.J. (2007). Epigenetic reversion of breast carcinoma phenotype is accompanied by changes in DNA sequestration as measured by AluI restriction enzyme. Am J Pathol *170*, 1739-1749.

Sankala, H., Vaughan, C., Wang, J., Deb, S., and Graves, P.R. (2011). Upregulation of the mitochondrial transport protein, Tim50, by mutant p53 contributes to cell growth and chemoresistance. Arch Biochem Biophys *512*, 52-60.

Sarig, R., Rivlin, N., Brosh, R., Bornstein, C., Kamer, I., Ezra, O., Molchadsky, A., Goldfinger, N., Brenner, O., and Rotter, V. (2010). Mutant p53 facilitates somatic cell reprogramming and augments the malignant potential of reprogrammed cells. J Exp Med *207*, 2127-2140.

Sato, R. (2010). Sterol metabolism and SREBP activation. Arch Biochem Biophys *501*, 177-181.

Schafer, Z.T., Grassian, A.R., Song, L., Jiang, Z., Gerhart-Hines, Z., Irie, H.Y., Gao, S., Puigserver, P., and Brugge, J.S. (2009). Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. Nature *461*, 109-113.

Scian, M.J., Stagliano, K.E., Anderson, M.A., Hassan, S., Bowman, M., Miles, M.F., Deb, S.P., and Deb, S. (2005). Tumor-derived p53 mutants induce NF-kappaB2 gene expression. Mol Cell Biol *25*, 10097-10110.

Scian, M.J., Stagliano, K.E., Deb, D., Ellis, M.A., Carchman, E.H., Das, A., Valerie, K., Deb, S.P., and Deb, S. (2004). Tumor-derived p53 mutants induce oncogenesis by transactivating growth-promoting genes. Oncogene *23*, 4430-4443.

Sepehrnia, B., Paz, I.B., Dasgupta, G., and Momand, J. (1996). Heat shock protein 84 forms a complex with mutant p53 protein predominantly within a cytoplasmic compartment of the cell. Journal of Biological Chemistry *271*, 15084-15090.

Shachaf, C.M., Perez, O.D., Youssef, S., Fan, A.C., Elchuri, S., Goldstein, M.J., Shirer, A.E., Sharpe, O., Chen, J., Mitchell, D.J., *et al.* (2007). Inhibition of HMGcoA reductase by atorvastatin prevents and reverses MYC-induced lymphomagenesis. Blood *110*, 2674-2684.

Shaw, K.R., Wrobel, C.N., and Brugge, J.S. (2004). Use of three-dimensional basement membrane cultures to model oncogene-induced changes in mammary epithelial morphogenesis. J Mammary Gland Biol Neoplasia *9*, 297-310.

Shaw, L.M., Rabinovitz, I., Wang, H.H., Toker, A., and Mercurio, A.M. (1997). Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. Cell *91*, 949-960.

Shibata, M.A., Ito, Y., Morimoto, J., and Otsuki, Y. (2004). Lovastatin inhibits tumor growth and lung metastasis in mouse mammary carcinoma model: a p53-independent mitochondrial-mediated apoptotic mechanism. Carcinogenesis *25*, 1887-1898.

Shibata, M.A., Kavanaugh, C., Shibata, E., Abe, H., Nguyen, P., Otsuki, Y., Trepel, J.B., and Green, J.E. (2003). Comparative effects of lovastatin on mammary and prostate oncogenesis in transgenic mouse models. Carcinogenesis *24*, 453-459.

Shvarts, A., Steegenga, W.T., Riteco, N., van Laar, T., Dekker, P., Bazuine, M., van Ham, R.C., van der Houven van Oordt, W., Hateboer, G., van der Eb, A.J., *et al.* (1996). MDMX: a novel p53-binding protein with some functional properties of MDM2. EMBO J *15*, 5349-5357.

Sigal, A., and Rotter, V. (2000). Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res *60*, 6788-6793.

Simpson, L., and Parsons, R. (2001). PTEN: life as a tumor suppressor. Exp Cell Res *264*, 29-41.

Singer, S., Ehemann, V., Brauckhoff, A., Keith, M., Vreden, S., Schirmacher, P., and Breuhahn, K. (2007). Protumorigenic overexpression of stathmin/Op18 by gain-of-function mutation in p53 in human hepatocarcinogenesis. Hepatology *46*, 759-768.

Siperstein, M.D. (1970). Cholesterol and cancer. Trans Am Clin Climatol Assoc *81*, 107-118.

Song, H., Hollstein, M., and Xu, Y. (2007). p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. Nat Cell Biol *9*, 573-580.

Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., *et al.* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A *98*, 10869-10874.

Soussi, T., and Lozano, G. (2005). p53 mutation heterogeneity in cancer. Biochem Biophys Res Commun *331*, 834-842.

Srivastava, S., Zou, Z.Q., Pirollo, K., Blattner, W., and Chang, E.H. (1990). Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature *348*, 747-749.

Staib, F., Hussain, S.P., Hofseth, L.J., Wang, X.W., and Harris, C.C. (2003). TP53 and liver carcinogenesis. Hum Mutat *21*, 201-216.

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., Shiff, I., Kogan, I., Shay, M., Kalo, E.*, et al.* (2010). Modulation of the vitamin D3 response by cancer-associated mutant p53. Cancer Cell *17*, 273-285.

Stein E A, L.P., Steiner P. (1993). Lovastatin 5-year safety and efficacy study. Lovastatin Study Groups I through IV. Arch Intern Med *153*, 1079-1087.

Stork, P.J., and Dillon, T.J. (2005). Multiple roles of Rap1 in hematopoietic cells: complementary versus antagonistic functions. Blood *106*, 2952-2961.

Strano, S., Dell'Orso, S., Di Agostino, S., Fontemaggi, G., Sacchi, A., and Blandino, G. (2007). Mutant p53: an oncogenic transcription factor. Oncogene *26*, 2212-2219.

Strano, S., Fontemaggi, G., Costanzo, A., Rizzo, M.G., Monti, O., Baccarini, A., Del Sal, G., Levrero, M., Sacchi, A., Oren, M.*, et al.* (2002). Physical interaction with human tumor-derived p53 mutants inhibits p63 activities. J Biol Chem *277*, 18817-18826.

Strano, S., Munarriz, E., Rossi, M., Castagnoli, L., Shaul, Y., Sacchi, A., Oren, M., Sudol, M., Cesareni, G., and Blandino, G. (2001). Physical interaction with Yes-associated protein enhances p73 transcriptional activity. J Biol Chem *276*, 15164-15173.

Strauss, B.E., and Haas, M. (1995). The region 3' to the major transcriptional start site of the MDR1 downstream promoter mediates activation by a subset of mutant P53 proteins. Biochem Biophys Res Commun *217*, 333-340.

Sun, T., Aceto, N., Meerbrey, K.L., Kessler, J.D., Zhou, C., Migliaccio, I., Nguyen, D.X., Pavlova, N.N., Botero, M., Huang, J.*, et al.* (2011). Activation of multiple proto-oncogenic tyrosine kinases in breast cancer via loss of the PTPN12 phosphatase. Cell *144*, 703-718.

Sun, Y., Cheung, J.M., Martel-Pelletier, J., Pelletier, J.P., Wenger, L., Altman, R.D., Howell, D.S., and Cheung, H.S. (2000). Wild type and mutant p53 differentially regulate the gene expression of human collagenase-3 (hMMP-13). J Biol Chem *275*, 11327-11332.

Sutter, A.P., Maaser, K., Hopfner, M., Huether, A., Schuppan, D., and Scherubl, H. (2005). Cell cycle arrest and apoptosis induction in hepatocellular carcinoma cells by HMG-CoA reductase inhibitors. Synergistic antiproliferative action with ligands of the peripheral benzodiazepine receptor. J Hepatol *43*, 808-816.

Suzuki, S., Tanaka, T., Poyurovsky, M.V., Nagano, H., Mayama, T., Ohkubo, S., Lokshin, M., Hosokawa, H., Nakayama, T., Suzuki, Y.*, et al.* (2010). Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. Proc Natl Acad Sci U S A *107*, 7461-7466.

Terzian, T., Suh, Y.A., Iwakuma, T., Post, S.M., Neumann, M., Lang, G.A., Van Pelt, C.S., and Lozano, G. (2008). The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. Genes Dev *22*, 1337-1344.

Teufel, D.P., Freund, S.M., Bycroft, M., and Fersht, A.R. (2007). Four domains of p300 each bind tightly to a sequence spanning both transactivation subdomains of p53. Proc Natl Acad Sci U S A *104*, 7009-7014.

Tobert, J.A. (2003). Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. Nat Rev Drug Discov *2*, 517-526.

Toledo, F., Krummel, K.A., Lee, C.J., Liu, C.W., Rodewald, L.W., Tang, M., and Wahl, G.M. (2006). A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network. Cancer Cell *9*, 273-285.

Toyooka, S., Tsuda, T., and Gazdar, A.F. (2003). The TP53 gene, tobacco exposure, and lung cancer. Hum Mutat *21*, 229-239.

Vance, D.E., and Van den Bosch, H. (2000). Cholesterol in the year 2000. Biochim Biophys Acta *1529*, 1-8.

Vargo-Gogola, T., and Rosen, J.M. (2007). Modelling breast cancer: one size does not fit all. Nat Rev Cancer *7*, 659-672.

Varley, J.M., Evans, D.G., and Birch, J.M. (1997). Li-Fraumeni syndrome--a molecular and clinical review. Br J Cancer *76*, 1-14.

Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., *et al.* (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science *303*, 844-848.

Venot, C., Maratrat, M., Sierra, V., Conseiller, E., and Debussche, L. (1999). Definition of a p53 transactivation function-deficient mutant and characterization of two independent p53 transactivation subdomains. Oncogene *18*, 2405-2410.

Vitolo, M.I., Weiss, M.B., Szmacinski, M., Tahir, K., Waldman, T., Park, B.H., Martin, S.S., Weber, D.J., and Bachman, K.E. (2009). Deletion of PTEN promotes tumorigenic signaling, resistance to anoikis, and altered response to chemotherapeutic agents in human mammary epithelial cells. Cancer Res *69*, 8275-8283.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.

Vousden, K.H., and Lu, X. (2002). Live or let die: the cell's response to p53. Nat Rev Cancer *2*, 594-604.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Vousden, K.H., and Ryan, K.M. (2009). p53 and metabolism. Nat Rev Cancer *9*, 691-700.

Walker, K.K., and Levine, A.J. (1996). Identification of a novel p53 functional domain that is necessary for efficient growth suppression. Proc Natl Acad Sci U S A *93*, 15335-15340.

Wang, F., Hansen, R.K., Radisky, D., Yoneda, T., Barcellos-Hoff, M.H., Petersen, O.W., Turley, E.A., and Bissell, M.J. (2002). Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. J Natl Cancer Inst *94*, 1494-1503.

Wang, F., Weaver, V.M., Petersen, O.W., Larabell, C.A., Dedhar, S., Briand, P., Lupu, R., and Bissell, M.J. (1998). Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc Natl Acad Sci U S A *95*, 14821-14826.

Wang, X., Sato, R., Brown, M.S., Hua, X., and Goldstein, J.L. (1994). SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell *77*, 53-62.

Warburg, O. (1956). On the origin of cancer cells. Science *123*, 309-314.

Warburg, O., Wind, F., and Negelein, E. (1927). The Metabolism of Tumors in the Body. J Gen Physiol *8*, 519-530.

Warner, E. (2011). Clinical practice. Breast-cancer screening. N Engl J Med *365*, 1025-1032.

Waterham, H.R. (2006). Defects of cholesterol biosynthesis. FEBS Lett *580*, 5442-5449.

Weaver, V.M., Lelievre, S., Lakins, J.N., Chrenek, M.A., Jones, J.C., Giancotti, F., Werb, Z., and Bissell, M.J. (2002). beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell *2*, 205-216.

Weaver, V.M., Petersen, O.W., Wang, F., Larabell, C.A., Briand, P., Damsky, C., and Bissell, M.J. (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol *137*, 231-245.

Weinberg, R.A. (1991). Tumor suppressor genes. Science *254*, 1138-1146.

Weisz, L., Damalas, A., Liontos, M., Karakaidos, P., Fontemaggi, G., Maor-Aloni, R., Kalis, M., Levrero, M., Strano, S., Gorgoulis, V.G., *et al.* (2007a). Mutant p53 enhances nuclear factor kappaB activation by tumor necrosis factor alpha in cancer cells. Cancer Res *67*, 2396-2401.

Weisz, L., Oren, M., and Rotter, V. (2007b). Transcription regulation by mutant p53. Oncogene *26*, 2202-2211.

Weisz, L., Zalcenstein, A., Stambolsky, P., Cohen, Y., Goldfinger, N., Oren, M., and Rotter, V. (2004). Transactivation of the EGR1 gene contributes to mutant p53 gain of function. Cancer Res *64*, 8318-8327.

Werner, H., Karnieli, E., Rauscher, F.J., and LeRoith, D. (1996). Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptor gene. Proc Natl Acad Sci U S A *93*, 8318-8323.

Whyte, J., Thornton, L., McNally, S., McCarthy, S., Lanigan, F., Gallagher, W.M., Stein, T., and Martin, F. (2010). PKCzeta regulates cell polarisation and proliferation restriction during mammary acinus formation. J Cell Sci *123*, 3316-3328.

Will, K., Warnecke, G., Wiesmuller, L., and Deppert, W. (1998). Specific interaction of mutant p53 with regions of matrix attachment region DNA elements (MARs) with a high potential for base-unpairing. Proc Natl Acad Sci U S A *95*, 13681-13686.

Wiman, K.G. (2010). Pharmacological reactivation of mutant p53: from protein structure to the cancer patient. Oncogene *29*, 4245-4252.

Wolf, D., Harris, N., and Rotter, V. (1984). Reconstitution of p53 expression in a nonproducer Ab-MuLV-transformed cell line by transfection of a functional p53 gene. Cell *38*, 119-126.

Wolf, D., and Rotter, V. (1984). Inactivation of p53 gene expression by an insertion of Moloney murine leukemia virus-like DNA sequences. Mol Cell Biol *4*, 1402-1410.

Wolf, J.K., Bodurka, D.C., Gano, J.B., Deavers, M., Ramondetta, L., Ramirez, P.T., Levenback, C., and Gershenson, D.M. (2004). A phase I study of Adp53 (INGN 201; ADVEXIN) for patients with platinum- and paclitaxel-resistant epithelial ovarian cancer. Gynecol Oncol *94*, 442-448.

Wong, K.B., DeDecker, B.S., Freund, S.M., Proctor, M.R., Bycroft, M., and Fersht, A.R. (1999). Hot-spot mutants of p53 core domain evince characteristic local structural changes. Proc Natl Acad Sci U S A *96*, 8438-8442.

Wong, W.W., Dimitroulakos, J., Minden, M.D., and Penn, L.Z. (2002). HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia *16*, 508-519.

Wrobel, C.N., Debnath, J., Lin, E., Beausoleil, S., Roussel, M.F., and Brugge, J.S. (2004). Autocrine CSF-1R activation promotes Src-dependent disruption of mammary epithelial architecture. J Cell Biol *165*, 263-273.

Xian, W., Pappas, L., Pandya, D., Selfors, L.M., Derksen, P.W., de Bruin, M., Gray, N.S., Jonkers, J., Rosen, J.M., and Brugge, J.S. (2009). Fibroblast growth factor receptor 1-transformed mammary epithelial cells are dependent on RSK activity for growth and survival. Cancer Res *69*, 2244-2251.

Xu, J., Reumers, J., Couceiro, J.R., De Smet, F., Gallardo, R., Rudyak, S., Cornelis, A., Rozenski, J., Zwolinska, A., Marine, J.C., *et al.* (2011). Gain of function of mutant p53 by coaggregation with multiple tumor suppressors. Nat Chem Biol *7*, 285-295.

Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature *445*, 656-660.

Yan, W., and Chen, X. (2009). Identification of GRO1 as a critical determinant for mutant p53 gain of function. J Biol Chem *284*, 12178-12187.

Yan, W., and Chen, X. (2010). Characterization of functional domains necessary for mutant p53 gain of function. J Biol Chem *285*, 14229-14238.

Yang, J., Richards, J., Guzman, R., Imagawa, W., and Nandi, S. (1980). Sustained growth in primary culture of normal mammary epithelial cells embedded in collagen gels. Proc Natl Acad Sci U S A *77*, 2088-2092.

Yoshida, Y., Kawata, M., Katayama, M., Horiuchi, H., Kita, Y., and Takai, Y. (1991). A geranylgeranyltransferase for rhoA p21 distinct from the farnesyltransferase for ras p21S. Biochem Biophys Res Commun *175*, 720-728.

Yoshikawa, K., Hamada, J., Tada, M., Kameyama, T., Nakagawa, K., Suzuki, Y., Ikawa, M., Hassan, N.M., Kitagawa, Y., and Moriuchi, T. (2010). Mutant p53 R248Q but not R248W enhances in vitro invasiveness of human lung cancer NCI-H1299 cells. Biomed Res *31*, 401-411.

Zhan, L., Rosenberg, A., Bergami, K.C., Yu, M., Xuan, Z., Jaffe, A.B., Allred, C., and Muthuswamy, S.K. (2008). Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. Cell *135*, 865-878.

Zhang, Y., Yan, W., and Chen, X. (2011). Mutant p53 disrupts MCF-10A cell polarity in 3-dimensional culture via epithelial-to-mesenchymal transitions. J Biol Chem.

Zhong, W.B., Liang, Y.C., Wang, C.Y., Chang, T.C., and Lee, W.S. (2005). Lovastatin suppresses invasiveness of anaplastic thyroid cancer cells by inhibiting Rho geranylgeranylation and RhoA/ROCK signaling. Endocr Relat Cancer *12*, 615-629.

Zhou, W., Muggerud, A.A., Vu, P., Due, E.U., Sorlie, T., Borresen-Dale, A.L., Warnberg, F., and Langerod, A. (2009). Full sequencing of TP53 identifies identical mutations within in situ and invasive components in breast cancer suggesting clonal evolution. Mol Oncol.

Zhu, J., Zhou, W., Jiang, J., and Chen, X. (1998). Identification of a novel p53 functional domain that is necessary for mediating apoptosis. J Biol Chem *273*, 13030-13036.

Zutter, M.M., Santoro, S.A., Staatz, W.D., and Tsung, Y.L. (1995). Re-expression of the alpha 2 beta 1 integrin abrogates the malignant phenotype of breast carcinoma cells. Proc Natl Acad Sci U S A *92*, 7411-7415.

FIGURE LEGENDS

Figure 1.1 *TP53* Mutational Spectrum in Human Breast Cancer

(A) *TP53* missense mutation data for human breast cancer patients (N=2,207) was obtained from the p53 IARC database and plotted as a function of amino acid position. Schematic of the p53 protein with domain structures illustrated. TAD: Transactivation Domain (1-42); PRD: Proline-Rich Domain (40-92), which also contains a second transactivation domain; DBD: DNA-Binding Domain (101-306); OD: Oligomerization Domain 307-355), also contains a nuclear export signal; CTD: C-Terminal Regulatory Domain (356-393), also contains three nuclear localization signals (*adapted from* p53.free.fr)

Figure 1.2 Schematic of Normal Acinar Development

(A) When non-malignant mammary epithelial cells are plated as single cells in a laminin-rich extracellular matrix (left), they first proliferate to form a spheroid. When the cells in the center of the spheroid lose contact with the basement membrane, they are induced to undergo apoptosis, leaving a single layer of apicobasally polarized cells surrounding a hollow lumen (right).

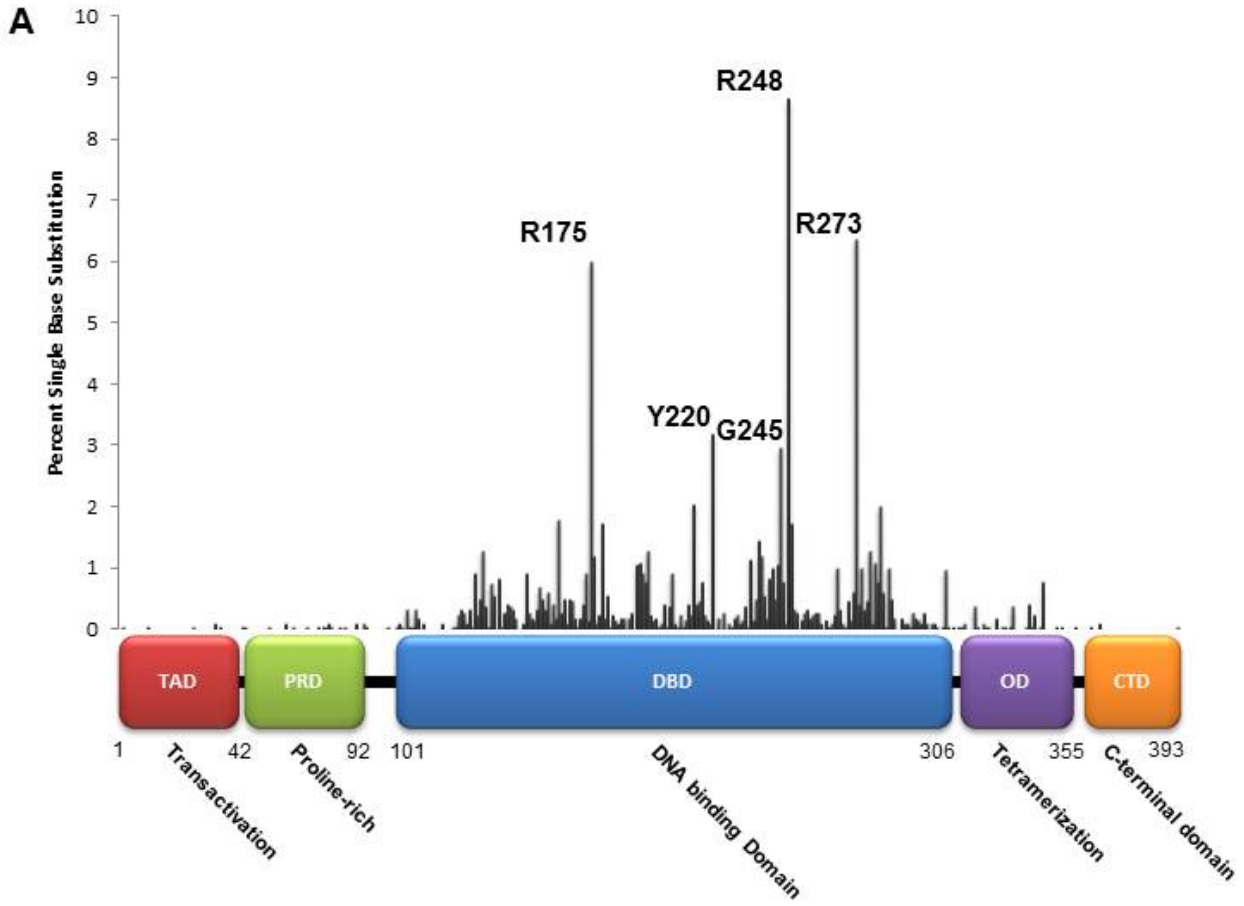
Table 1.1 Select list of mutant p53 transcriptionally-activated genes

A literature search was performed for mutant p53 transcriptionally-activated genes. Genes were grouped into biological categories based on reported functions and Gene Ontology annotations.

Table 1.2 Select list of proteins implicated in 3D acinar morphogenesis

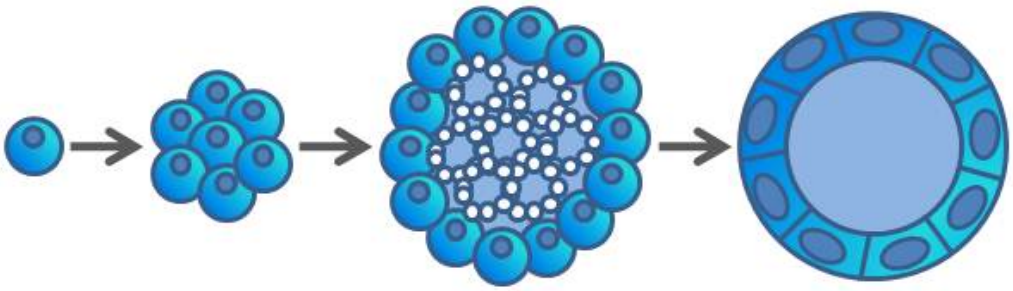
A literature search was performed for proteins/pathways reported to disrupt acinar morphogenesis or maintain the malignant state of tumorigenic breast cells.

Freed-Pastor et al., Figure 1.1



Freed-Pastor et al., Figure 1.2

A



Freed-Pastor et al., Table 1.1

Category	Gene Symbol	Name/Synonym	Mutant(s)	Reference(s)
Increased Proliferation	*MYC*	c-Myc	V143A, R175H, R273H, R248W, D281G	(Frazier et al., 1998)
	CXCL1	Chemokine (C-X-C motif) ligand 1; GRO1	R175H, R273H, R248W, G245S	(Yan and Chen, 2009, 2010)
	MAP2K3	Mitogen activated protein kinase kinase 3	R175H, R273H, R280K	(Bossi et al., 2008; Gurtner et al., 2010)
	FOS	c-Fos	C174Y	(Preuss et al., 2000)
	PCNA	Proliferating cell nuclear antigen	V143A, R175H, R248W, R273H, D281G	(Deb et al., 1992)
	MAD1L1	MAD1 mitotic arrest deficient-like 1	D281G	(Iwanaga and Jeang, 2002)
	CCNE2	Cyclin E2	R280K, R273H	(Girardini et al., 2011)
	CCNA2	Cyclin A2	R175H, L194F, R273H	(Di Agostino et al., 2006)
	CCNB1	Cyclin B1	R175H, L194F, R273H	(Di Agostino et al., 2006)
	CCNB2	Cyclin B2	R175H, L194F, R273H, D281G	(Di Agostino et al., 2006)
	CDK1	Cyclin dependent kinase 1	R175H, L194F, R273H	(Di Agostino et al., 2006)
	CDC25C	Cell division cycle 25 homolog C	R175H, L194F, R273H, D281G	(Di Agostino et al., 2006)
	E2F5	E2F5	D281G	(Scian et al., 2005)
	ASNS	Asparagine Synthetase	R175H, R273H, D281G	(Scian et al., 2004)
	IGF1R	Insulin-like Growth Factor 1 Receptor	R175H, R248W, R273H	(Bossi et al., 2008; Werner et al., 1996)
	IGFBP3	Insulin-like growth factor binding protein 3	R175H	(Stambolsky et al., 2010)
	EGFR	Epidermal Growth Factor Receptor	V143A, R175H, R248W, R273H, D281G	(Ludes-Meyers et al., 1996)
	MCM6	Minichromosome maintenance complex component 6	D281G	(Scian et al., 2005)
	STMN1	Stathmin 1	Y220C, R213Q	(Singer et al., 2007)
Inhibition of Cell Death	*EGR1*	Early growth response 1	R175H, H179E, R248W, R273H, D281G	(Weisz et al., 2004)
	NFKB2	NF-κB (p52)	R175H, R273H, D281G	(Scian et al., 2005)
	ABCB1	ATP-binding cassette sub-	R175H, R248Q,	(Chin et al., 1992;

		family B member 1; MDR1	D281G	Lin et al., 1995; Sampath et al., 2001; Strauss and Haas, 1995)
	TIMM50	Translocator of the inner mitochondrial membrane 50	R175H, R273H	(Sankala et al., 2011)
	LGALS3	Lectin, galactoside-binding, soluble; Galectin-3	R273H	(Lavra et al., 2009)
	BCL2L1	Bcl-xL	R273H	(Bossi et al., 2008)
	IGF2	Insulin-like growth factor 2	R249S	(Lee et al., 2000)
	DUT	Deoxyuridine triphosphatase; dUTPase	R175H, R248W	(Pugacheva et al., 2002)
Metabolism	*DHCR24*	24-dehydrocholesterol reductase; Seladin-1	R175H, R273H	(Bossi et al., 2008)
	CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1	R175H, R273H	(Stambolsky et al., 2010)
Cell-cell /Cell-ECM Signaling	*MMP3*	Matrix metalloproteinase 3	R175H	(Buganim et al., 2010)
	MMP13	Matrix metalloproteinase 13	R175H, D281G	(Sun et al., 2000)
	ITGA6	Integrin alpha 6	D281G	(Scian et al., 2005)
	PXN	Paxillin beta	R175H, R273H	(Bossi et al., 2008)
GTPase activity	*ARHGDIA*	Rho GDP dissociation inhibitor alpha	R175H, R273H	(Bossi et al., 2008)
	RANGAP1	Ran GTPase activating protein 1	R175H, R273H	(Bossi et al., 2008)
	DEPDC1	DEP domain containing 1	R280K, R273H	(Girardini et al., 2011)
	WDR67	WD repeat domain 67	R280K, R273H	(Girardini et al., 2011)
Limitless replication	*TERT*	Telomerase reverse transcriptase	D281G	(Scian et al., 2004)
Cytoskeleton	*KIF20A*	Kinesin family member 20A; *RAB6KIFL*	R175H, R273H	(Bossi et al., 2008)
Cytoskeleton	*EPB41L4B*	Erythrocyte membrane protein band 4.1 like 4B	R280K, R273H	(Girardini et al., 2011)
M-phase	*BUB1*	Budding uninhibited by benzimidadazoles 1 homolog	R280K, R273H	(Girardini et al., 2011)
Centromere	*NCAPH*	Non-SMC condensing I complex, subunit H	R280K, R273H	(Girardini et al., 2011)
Centromere	*MIS18A*	MIS18 kinetochore protein homolog A; C21orf45	R280K, R273H	(Girardini et al., 2011)
Centromere	*CENPA*	Centromere protein A	R280K, R273H	(Girardini et al., 2011)
mRNA processing	*CPSF6*	Cleavage and polyadenylation specific factor 6	R280K, R273H	(Girardini et al., 2011)
Unknown	*FAM64A*	Family with sequence similarity 64, member A	R280K, R273H	(Girardini et al., 2011)
RNA stability	*ID4*	Inhibitor of DNA binding 4	R175H, R273H	(Fontemaggi et al., 2009)

Freed-Pastor et al., Table 1.2

Category	Protein(s)	Disrupt Acinar Morphogenesis	Phenotypic Reversion	Reference(s)
Extracellular	MMP-3	Yes (OE: SCp2)	N.D.	(Lochter et al., 1997)
	MMP-9	Yes (OE: S1, MCF10A)	Yes (KD/inh: T4-2)	(Beliveau et al., 2010)
	ADAM17 (TACE-1)	N.D.	Yes (KD/inh: T4-2)	(Kenny and Bissell, 2007)
	TIMP-1	Yes (OE: MCF10A)	N.D.	(Jung et al., 2006; Liu et al., 2005)
	Fibronectin	N.D.	Yes (Inh: T4-2)	(Sandal et al., 2007)
Cell Adhesion	Integrin β1	N.D.	Yes (Inh: T4-2)	(Wang et al., 2002; Wang et al., 1998; Weaver et al., 1997)
	Integrin β4	Yes (Inh: S1)	Yes (KD/inh: SUM159)	(Gabarra et al., 2010; Lipscomb et al., 2005; Weaver et al., 1997)
	Integrin α2	N.D.	Yes (OE: Mm5MT)	(Zutter et al., 1995)
	Dystroglycan	N.D.	Yes (OE: T4-2)	(Muschler et al., 2002)
	E-Cadherin	Yes (KD: S1)	Yes (OE: SKBR3, MDA-231)	(Fournier et al., 2009; Meiners et al., 1998; Wang et al., 2002)
	CEACAM1	Yes (KD: MCF10F)	Yes (OE: MCF7)	(Huang et al., 1999)
Receptor Tyrosine Kinases (RTKs)	CSF-1/CSF-1R	Yes (OE: MCF10A)	N.D.	(Wrobel et al., 2004)
	c-Met/HGF	Yes (OE: MCF10A)	N.D.	(Wrobel et al., 2004)
	ErbB2 (HER2/neu)	Yes (OE: MCF10A)	N.D.	(Arias-Romero et al., 2010; Muthuswamy et al., 2001)
	ErbB1 (EGFR)	No (OE: MCF10A)	Yes (Inh: T4-2)	(Beliveau et al., 2010; Itoh et al.,

				2007; Muthuswamy et al., 2001; Wang et al., 2002; Wang et al., 1998)
	IGF1R	Yes (OE: MCF10A)	N.D.	(Irie et al., 2005; Irie et al., 2010; Litzenburger et al., 2009)
	FGFR1	Yes (OE: MCF10A)	N.D.	(Xian et al., 2009)
	PTPN12	Yes (KD: MCF10A)	N.D.	(Sun et al., 2011)
Intracellular signaling	PI3K	Yes (OE: MCF10A): *PIK3CA* mutants	Yes (Inh: T4-2)	(Beliveau et al., 2010; Isakoff et al., 2005; Liu et al., 2004b; Wang et al., 2002)
	Akt	Yes (OE: MCF10A)	N.D.	(Debnath et al., 2003b)
	PTEN	Yes (KD: S1)*	N.D.	(Fournier et al., 2009)
	Raf	Yes (OE: S1, MCF10A)	N.D.	(Beliveau et al., 2010; Herr et al., 2011)
	MEK-1/2 (p42/p44 MAPK)	N.D.	Yes (Inh: T4-2)	(Wang et al., 2002; Wang et al., 1998)
	Rap-1	N.D.	Yes (DN: T4-2)	(Itoh et al., 2007)
	Src	Yes (OE: MCF10A)	N.D.	(Reginato et al., 2005)
	Scribble	Yes (KD: MCF10A)	N.D.	(Zhan et al., 2008)
	PKC-zeta (aPKC)	Yes (DN: MCF10A)	N.D.	(Whyte et al., 2010)
Nuclear Factors	BRCA1	Yes (KD: MCF10A)	N.D.	(Furuta et al., 2005)
	p53 (mutant)	Yes (OE: MCF10A)	N.D.	(Zhang et al., 2011)
	Yes-Associated Protein (YAP)	Yes (OE: MCF10A)	N.D.	(Overholtzer et al., 2006)
	HOXD10	N.D.	Yes (OE: MDA-	(Carrio et al.,

		231)	2005)
	TACC2 (AZU-1)	N.D.	Yes (OE: T4-2)	(Chen et al., 2000)
*However PTEN homozygous deletion from MCF10A cells does not affect acinar morphogenesis (Vitolo et al., 2009)				
OE=overexpression; KD=knockdown via RNAi; Inh=inhibition by specific inhibitors or inhibitory antibodies; DN=dominant negative.				

Chapter 2

Mutant p53 Disrupts Mammary Acinar Morphogenesis via the Mevalonate Pathway

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SUMMARY

p53 is a frequent target for mutation in human tumors and mutant p53 proteins can actively contribute to tumorigenesis. We employed a three-dimensional culture model in which non-malignant breast epithelial cells form spheroids reminiscent of acinar structures found *in vivo*, whereas breast cancer cells display highly disorganized morphology. We found that mutant p53 depletion is sufficient to phenotypically revert breast cancer cells to a more acinar-like morphology. Genome-wide expression analysis identified the mevalonate pathway as significantly upregulated by mutant p53. Statins and sterol biosynthesis intermediates reveal that this pathway is both necessary and sufficient for the phenotypic effects of mutant p53 on breast tissue architecture. Mutant p53 associates with sterol gene promoters at least partly via SREBP transcription factors. Finally, p53 mutation correlates with highly expressed sterol biosynthesis genes in human breast tumors. These findings provide insight into breast carcinogenesis and implicate the mevalonate pathway as a therapeutic target for tumors bearing mutations in p53.

HIGHLIGHTS

- Depletion of mutant p53 phenotypically reverts breast cancer cells in 3D culture
- Mutant p53 upregulates 17 genes encoding enzymes in the mevalonate pathway
- HMG-CoA reductase inhibitors mimic the phenotypic effects of mutant p53 depletion
- *TP53* mutation correlates with high levels of mevalonate pathway genes in human tumors

INTRODUCTION

The *TP53* gene, which encodes the p53 protein, is the most frequent target for mutation in tumors, with over half of all human cancers exhibiting mutation at this locus (Vogelstein et al., 2000). Wild-type p53 functions primarily as a transcription factor and possesses an N-terminal transactivation domain, a centrally located sequence specific DNA binding domain, followed by a tetramerization domain and a C-terminal regulatory domain (Laptenko and Prives, 2006). In response to a number of stressors, including DNA damage, hypoxia and oncogenic activation, p53 becomes activated to promote cell cycle arrest, apoptosis or senescence thereby suppressing tumor growth. It also plays many additional roles including regulating cellular metabolism (Vousden and Prives, 2009).

Unlike most tumor suppressor genes, which are predominantly inactivated as a result of deletion or truncation, the majority of mutations in *TP53* are missense mutations, a few of which cluster at "hotspot" residues in the DNA binding core domain (Petitjean et al., 2007), while the N- and C-terminal domains of this protein are relatively spared from mutation (Hussain and Harris, 1998; Soussi and Lozano, 2005; Unger et al., 1993). In contrast to wild-type p53, which under unstressed conditions is a very short-lived protein, these missense mutations lead to the production of full-length p53 protein with a prolonged half-life (Brosh and Rotter, 2009; Davidoff et al., 1991; Rotter, 1983). While many tumor-derived mutant forms of p53 can exert a dominant-negative effect on the remaining wild-type allele, serving to abrogate the ability of wild-type p53 to inhibit cellular transformation, the end result in many forms of human cancer is frequently loss of heterozygosity (LOH), where the wild-type version of p53 is lost and the mutant form is retained, suggesting that there is a selective advantage conferred by losing the remaining wild-type p53, even after one allele has been mutated (Brosh and Rotter, 2009).

There is substantial evidence that mutant forms of p53 can exert oncogenic, or gain-of-function, activities independent of their effects on wild-type p53. *In vivo* models, in which mice harboring two tumor-derived mutants of p53 (equivalent to R175H and R273H in humans) that were substituted for the endogenous wild-type p53 locus within the mouse genome, display an altered tumor spectrum as well as more metastatic tumors (Lang et al., 2004; Olive et al., 2004). The mutational status of p53 has been shown to predict poor outcomes in multiple types of human tumors, including breast cancer, and certain p53 mutants associate with an even worse prognosis (Olivier et al., 2006; Petitjean et al., 2007). Mutant p53 has also been demonstrated to lead to increased survival, invasion, migration and metastasis in preclinical breast cancer models (Adorno et al., 2009; Muller et al., 2009; Stambolsky et al., 2010). Despite these findings, mutant p53-induced phenotypic alterations in mammary tissue architecture have not been fully explored.

Breast cancer is thought to arise from mammary epithelial cells found in structures referred to as acini, which collectively form terminal ductal lobular units (TDLU). Each acinus consists of a single layer of polarized luminal epithelial cells surrounding a hollow lumen (Allred et al., 2001; Bissell et al., 2002). While traditional "2D" cell culture has provided insight into the process of breast carcinogenesis, such *in vitro* culture conditions create an environment that markedly differs from the microenvironment that a cell would experience *in vivo* (Vargo-Gogola and Rosen, 2007). By contrast, normal mammary epithelial cells, when grown in a laminin-rich extracellular matrix, form three-dimensional structures highly reminiscent of many aspects of acinar structures found *in vivo* (Debnath et al., 2003; Petersen et al., 1992) and the processes and pathways that govern and disrupt normal mammary epithelial development in this setting have been elegantly defined (Debnath et al., 2002; Muthuswamy et al., 2001; Schafer et al., 2009;

Wrobel et al., 2004; Zhan et al., 2008). Since one of the hallmarks of breast tumorigenesis is the disruption of mammary tissue architecture (Friedrich, 2003), three-dimensional (3D) culture conditions allow one to readily distinguish normal and tumorigenic tissue by morphological phenotype (Kenny et al., 2007; Martin et al., 2008; Muthuswamy et al., 2001). In addition, inhibition of key oncogenic signaling pathways is sufficient to phenotypically revert breast cancer cells grown in 3D culture (Beliveau et al., 2010; Bissell et al., 2005; Wang et al., 1998; Weaver et al., 1997). In this study we implicate mutant p53 in the disruption of acinar morphology and identify a critical cellular pathway by which mutant p53 disrupts mammary tissue architecture. Specifically, we identified seventeen genes encoding enzymes within the mevalonate pathway, by which cells produce cholesterol and essential metabolites for a diverse set of cellular processes, as novel activation targets of mutant p53 in breast cancer. The mevalonate pathway has been previously implicated in multiple aspects of tumorigenesis (Cao et al., 2011; Clendening et al., 2010; Koyuturk et al., 2007; Shachaf et al., 2007; Wong et al., 2002) and this pathway is the target of the safe and effective class of statin drugs (Buchwald, 1992; Wong et al., 2002). Our data have also revealed a potential mechanism by which mutant p53 expression increases expression of the genes in the mevalonate pathway.

RESULTS

Mutant p53 depletion in breast cancer cells leads to a phenotypic reversion in 3D culture

To investigate the role of mutant p53 in breast cancer, we employed the 3D culture protocol where mammary epithelial cells are grown in a laminin rich extracellular matrix (Matrigel). In 3D culture, non-malignant MCF10A mammary cells with wild-type p53 undergo a well-characterized progression of three-dimensional morphogenesis, which results in spherical

acinus-like structures (Debnath et al., 2003; Petersen et al., 1992) (Figure 2.S2A). We examined the 3D morphologies of two cell lines derived from metastatic breast tumors that each expresses exclusively a single mutant p53 allele; MDA-231 (R280K) and MDA-468 (R273H). These cells were engineered to stably express a miR30-based doxycycline-inducible shRNA targeting endogenous mutant p53 in the 3' UTR (designated MDA-231.shp53 and MDA-468.shp53). In both cases mutant p53 reduction by shRNA led to dramatic changes in the behavior of the cells when cultured in a 3D microenvironment. MDA-231 cells, when grown in 3D culture, normally exhibit an extremely disordered and invasive morphology, which has been characterized as "stellate" (Kenny et al., 2007). Depleting these cells of mutant p53 in 3D culture conditions almost completely abrogated the stellate morphology of large, invasive structures with bridging projections (Figure 2.1A). Instead, MDA-231 cells with reduced p53 developed smaller, less invasive appearing cell clusters. By titrating doxycycline, we observed a progressive loss of malignant, invasive characteristics as a function of decreasing levels of mutant p53 (Figure 2.S1A-D). This reduction in invasive behavior in 3D culture supports the recent findings that mutant p53 promotes the invasion of breast cancer cells (Adorno et al., 2009; Muller et al., 2009). Nevertheless, when plated in 3D culture, MDA-231 cells with reduced p53 did not assume the ordered acinus-like morphology that is characteristic of non-malignant mammary epithelial cells.

MDA-468 cells have a less invasive, but highly disorganized appearance, and have been classified as "grape-like" rather than "stellate" (Kenny et al., 2007). Under 3D culture conditions, MDA-468.shp53 cells displayed three types of cellular morphologies (1) constellations of cells with a highly disordered "malignant" appearance that comprise about 30-40% of the population, (2) spherical cell clusters with an "intermediate" morphology that, while

disordered, appear less malignant (about 55-65% of the population) and (3) a very small proportion (<5%) of structures that closely resemble small acini and contain a hollow lumen (examples of these categories are shown in Figure 2.1C). Strikingly, when mutant p53 was depleted from these cells, a significant proportion of the population underwent a full phenotypic reversion from highly disorganized structures to acinus-like structures with a hollow lumen (Figure 2.1D). These reverted structures also display proper localization of alpha 6 integrin, suggesting that they have regained apicobasal polarization (Figure 2.S1E). Consistent with previous studies implicating programmed cell death in the process of luminal clearance (Debnath et al., 2002), we occasionally identified dying cells within the luminal space (Figure 2.1D; image in right panel reveals dying cell within the central luminal region). Using either a stable pool of MDA-468.shp53 cells (Figure 2.1F) or a stable clone derived from these cells (Figure 2.1G), we observed a significant increase in the hollow lumen population upon mutant p53 depletion, with nearly 50% of the population falling into this acinus-like morphology in the latter case. It is important to note that while in the experiment shown in Figure 2.1G, we observed a concomitant decrease in predominantly the intermediate population upon the reversion to hollow lumen structures, in other cases we observed a decrease in both the malignant and intermediate populations (e.g. Figure 2.4A below). Since the stable clone of MDA-468.shp53 cells exhibited a higher degree of reversion, all further experiments were carried out using these cells. Importantly, since both of these breast cancer cell lines express only mutant p53, these phenotypic changes may be attributed directly to the reduction in mutant p53 levels.

To confirm and expand upon these observations, we engineered MDA-468.shp53 cells to express an shRNA-resistant version of the p53 mutant that is endogenously found in these cells (p53-R273H) or a control vector (Figure 2.2A-B). Introducing excess mutant p53 into these

already malignant cells prevented the phenotypic reversion that normally occurs after depleting cells of mutant p53 (Figure 2.2B). In fact, exogenous mutant p53, combined with the endogenous level of mutant p53 led to an even more exaggerated malignant phenotype (highly disorganized and invasive) than parental cells (compare left panels of Figure 2.2A and 2B).

Wild-type p53 primarily functions as a transcription factor and the transactivation domains of p53 have previously been implicated in oncogenic functions of mutant p53 such as survival and resistance to chemotherapeutics (Lin et al., 1995; Matas et al., 2001; Yan and Chen, 2010). To interrogate the role of the transactivation domains in the effects of mutant p53 in 3D culture, we engineered MDA-468.shp53 cells to express an shRNA-resistant version of the endogenous mutant p53 that had been mutated at four key residues (L22Q/W23S/W53Q/F54S), shown previously to render its transactivation domains non-functional (Lin et al., 1994; Venot et al., 1999). As opposed to mutant p53 with a functional transactivation region (Figure 2.2B), the transactivation-dead version of mutant p53 failed to rescue the phenotypic reversion (Figure 2.2C), suggesting that the oncogenic effects in this system were due to transcriptional changes mediated by mutant p53.

In order to test whether the effects of mutant p53 on 3D morphology of breast cancer cells were generalizable between tumor-derived mutants of p53, we replaced the endogenous mutant p53 in MDA-231 cells (R280K) with an shRNA-resistant version of p53-R273H, the mutant that is endogenously expressed in MDA-468 cells. While control cells behaved like the cells with just the shRNA-targeting p53, expression of p53-R273H partially prevented the phenotypic changes of knocking down the endogenous p53-R280K (Figure 2.S1F). We also engineered the non-malignant human mammary epithelial cell line, MCF10A, to express Flag-tagged versions of the five most frequent p53 mutants found in breast tumors (p53-R175H, -

R248Q, -R273H, -R248W, -G245S) (http://p53.free.fr). MCF10A cells infected with a control vector exhibited normal acinar morphogenesis. However, in agreement with recently published findings (Zhang et al., 2011), expression of the four most frequent mutant p53 proteins led to an inhibition of luminal clearance, reminiscent of the filled lumen phenotype observed in ductal carcinoma in situ (DCIS) lesions, with p53-R273H and p53-R248W exhibiting the highest degree of luminal filling (Figure 2.S2B-F). To examine whether this phenotype was also dependent on the transactivation capacity of p53 mutants, we generated MCF10A cells expressing transactivation-deficient versions (mTAD) of these same five p53 mutants, which were unable to block luminal clearance, with p53-R273H and p53-R248W displaying the highest dependence on their transactivation domains (Figure 2.S2I-J). Thus, not only can depletion of mutant p53 from breast cancer cells lead to a phenotypic reversion in 3D culture, but also mutant p53 expression in non-malignant mammary epithelial cells is sufficient to disrupt their morphology in 3D culture and the transactivation domain of mutant p53 is required in both cases.

Tumor-derived mutants of p53 regulate the mevalonate pathway in breast cancer cells

Since the transactivation activity of mutant p53 is very likely to be critical for its phenotypic effects in 3D culture, we performed genome-wide expression profiling on MDA-468.shp53 cells grown in 3D culture, with or without mutant p53 knockdown. We identified 989 genes as significantly altered ($p<0.01$) following shRNA-mediated downregulation of endogenous mutant p53, suggesting that mutant p53 acts promiscuously to affect many cellular processes. To guide our identification of those pathways/processes necessary for mutant p53 function in 3D culture, we employed two analysis methods, Ingenuity Pathway Analysis (IPA) and Gene Ontology (GO) Analysis. Since each of these analysis tools has a unique approach for grouping genes according to the pathway or process in which their protein products are reported

to function, we exploited both techniques in hopes that pathways/processes that were identified using the two analyses would be more likely to have functional significance. The mevalonate pathway was the most overrepresented cellular pathway using IPA (labeled "Steroid Biosynthesis Pathway" by Ingenuity); in fact, it was the only pathway detected with 99% confidence ($p<0.01$) following mutant p53 downregulation (Figure 2.3A). This pathway, along with the related isoprenoid biosynthetic process, was also detected using GO analysis and was significantly downregulated upon mutant p53 ablation across three independent experiments (Figure 2.3B).

The mevalonate pathway is responsible for *de novo* cholesterol synthesis as well as for many important non-sterol isoprenoid derivatives (Figure 2.S3) (Goldstein and Brown, 1990). Of the many steps that convert Acetyl-CoA to cholesterol, seven genes (*HMGCR*, *MVK*, *MVD*, *FDPS*, *SQLE*, *LSS*, *DHCR7*) encoding enzymes within the mevalonate pathway were found to be significantly reduced by mutant p53 depletion according to the IPA. In separate experiments, using qRT-PCR we confirmed that expression of all of these genes were markedly reduced ($p<0.005$) when mutant p53 was depleted by shRNA (Figure 2.3C). We also confirmed that p53-mediated regulation of a subset of these genes in MDA-468 cells occurs as a result of RNA transcription (as opposed to mRNA stability or some later point of regulation) by using primers for nascent transcripts that anneal to intronic regions (data not shown). The genes that were affected by mutant p53 knockdown encode key enzymes throughout the mevalonate pathway (Figure 2.S3), including the rate-limiting enzyme, 3-Hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase).

The effects of mutant p53 on breast cancer morphology are mediated through the mevalonate pathway

Elevated or deregulated activity of the mevalonate pathway has been demonstrated in a number of different tumors, including breast cancer (Koyuturk et al., 2007; Wong et al., 2002), and high levels of many of the enzymes in this pathway have been shown to have prognostic significance in breast cancer (Clendening et al., 2010). This pathway was demonstrated to be necessary for DNA synthesis (Langan and Volpe, 1986; Quesney-Huneeus et al., 1979) and a number of studies have suggested that malignant cells are more highly dependent on the continuous availability of metabolites produced by the mevalonate pathway than their non-malignant counterparts (Buchwald, 1992; Larsson, 1996). While the mevalonate pathway has been explored most extensively in the context of cholesterol production, which is necessary for membrane integrity and thus cell division, many of the intermediate metabolites and side products play key roles in other essential cellular processes. For example, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are critical for post-translational modifications of Ras and RhoA, respectively (Casey et al., 1989; Fried, 2008; Yoshida et al., 1991). Cholesterol is necessary for the formation of steroid hormones, such as estrogen, progesterone and vitamin D (Goldstein and Brown, 1990). Coenzyme Q_{10} (ubiquinone) plays a vital role in cell respiration, dolichol is essential for N-linked glycosylation of proteins and mevalonic acid has been suggested to promote the cell cycle directly (Graaf et al., 2004; Quesney-Huneeus et al., 1979).

We took advantage of the fact that many of the biologically active intermediate enzymatic products produced by the mevalonate pathway can be readily taken up by cells in culture (Denoyelle et al., 2001). To test whether this pathway is necessary for the phenotypic effects of mutant p53, add-back experiments were performed in which breast cancer cells grown in 3D culture were depleted of mutant p53 and supplemented with intermediate metabolites

produced by the mevalonate pathway. Addition of the two earliest metabolites, mevalonic acid (MVA) and mevalonic acid phosphate (MVAP), was sufficient to dramatically inhibit the phenotypic reversion caused by mutant p53 knockdown in MDA-468 cells without affecting the amount of p53 depletion (Figure 2.4A and S4A-B). This confirms that activity of the mevalonate pathway is sufficient to compensate for the loss of mutant p53 and suggests that up-regulation of at least the initial steps of the mevalonate pathway is necessary for the effects of mutant p53 on tissue architecture.

HMG-CoA reductase, which catalyzes the formation of mevalonic acid, is the rate limiting step in cholesterol biosynthesis and is famously the target of numerous cholesterol reducing statins (Katz et al., 2005). The use of statins is well established in the clinic to treat patients with hypercholesterolemia and there have been multiple reports demonstrating that statins can exhibit anti-cancer activity; however, their anti-tumorigenic mechanism has not been firmly established (Campbell et al., 2006; Cao et al., 2011; Koyuturk et al., 2007; Shibata et al., 2003).

We hypothesized that pharmacologic inhibition of the rate-limiting enzyme in the mevalonate pathway might be sufficient to mimic the effects of knocking down mutant p53. Strikingly, we found that treatment of breast cancer cells in 3D culture with Simvastatin, a lipophilic statin, used at clinically achievable concentrations (Wong et al., 2002), resulted in a reduction in growth in both cell lines, in addition to extensive cell death in MDA-468 cells (Figure 2.4B) and a significant reduction of the invasive morphology of MDA-231 cells (Figure 2.4C). In fact, in MDA-231 cells the morphological changes seen with either statin treatment or mutant p53 knockdown were virtually the same. The consequence of inhibiting sterol biosynthesis in MDA-468 cells was even more dramatic than mutant p53 downregulation alone

(cell death as opposed to formation of structures with a hollow lumen). On the other hand, inhibition of HMG-CoA reductase in wild-type p53 expressing MCF10A cells did not result in gross morphologic changes when used at clinically achievable concentrations (Figure 2.S4C). This suggests that breast cancer cells bearing mutations in p53 upregulate the mevalonate pathway and eventually become dependent upon its activity for survival. Similar results were obtained with another lipophilic statin, Mevastatin (Figure 2.S4D and E). Importantly, supplementation of mevalonic acid, the enzymatic product of HMG-CoA reductase, to either MDA-468 or MDA-231 cells treated with a statin blocked many of the phenotypic effects of statins (Compare Figure 2.4D to 4B or 4C). These results indicate that the effects of statins on breast cancer cells in 3D culture occur because of the function of HMG-CoA reductase to produce mevalonic acid, and further implicate the upregulated mevalonate pathway in the malignant 3D phenotype of these cells. In addition, we tested whether flux through the mevalonate pathway was sufficient to disrupt normal acinar morphogenesis. We cultured MCF10A cells in 3D culture with or without supplementation of mevalonic acid (MVA) and demonstrate that, similar to overexpression of tumor-derived mutants of p53, exogenous mevalonic acid is sufficient to block luminal clearance in MCF10A cells (Figure 2.4E).

We extended the effects of statins on breast cancer cells in three other assays. First, we tested whether statin treatment had an impact on anchorage-independent growth and observed that Simvastatin can significantly impair anchorage-independent growth in both MDA-468 and MDA-231 cells (Figure 2.S5A). Second, inhibition of HMG-CoA reductase has previously been reported to induce cell cycle arrest and/or apoptosis in a variety of cell lines grown in traditional (2D) cell culture (Jakobisiak et al., 1991; Sanchez et al., 2008). In line with these findings, we noted a G1 cell cycle arrest, with a concomitant drop in S phase, in both breast cancer cell lines

treated with 24 hours of Simvastatin at varying concentrations (Figure 2.S5B-C). It is therefore likely that the observed phenotypic effects of statins in 3D culture are due to a combination of factors (i.e. decreased growth, increased death and decreased invasion). Third, in addition to these *ex vivo* experiments, we also examined whether statin-mediated inhibition of HMG-CoA reductase would have an effect on breast cancer cells *in vivo*. Using a xenograft model of MDA-231 breast cancer cells, we demonstrate that Simvastatin significantly impairs tumor growth of these cells when implanted into immunocompromised mice (Figure 2.S5D). This supports earlier studies that demonstrate that HMG-CoA reductase inhibition can impact tumor formation using breast cancer xenograft models (Ghosh-Choudhury et al., 2010; Mori et al., 2009).

Geranylgeranylation is required for the phenotypic effects of mutant p53 in MDA-231 breast cancer cells grown in 3D culture

Next we tested whether inhibition of later enzymes within the mevalonate pathway would have similar phenotypic effects as mutant p53 depletion from breast cancer cells grown in 3D culture. An inhibitor of Mevalonate Decarboxylase, 6-Fluoromevalonate (Cuthbert and Lipsky, 1990), had remarkably similar phenotypic effects on both MDA-468 and MDA-231 cells grown in 3D culture to that seen with inhibition of HMG-CoA reductase by statins (Figure 2.S4). Thus, not only HMG-CoA reductase, but several downstream enzymatic steps in the mevalonate pathway are involved in the ability of mutant p53 to prevent normal morphological behavior of breast cancer cells in 3D culture conditions.

Because the mevalonate pathway is not only vital for producing cellular cholesterol, but many other biologically active intermediate metabolites, we examined whether the phenotypic effects of mutant p53 knockdown were due to decreased cholesterol synthesis or the production

of an earlier metabolite. To do this, we utilized three inhibitors that inhibit distinct actions of the mevalonate pathway (Figure 2.S3). YM-53601 inhibits squalene synthase (and thus cholesterol production) at submicromolar concentrations (Ugawa et al., 2000), but spares all upstream intermediate metabolites. FTI-277 blocks farnesylation of proteins via inhibition of farnesyl transferase at nanomolar concentrations in whole cells, but has no effect on geranylgeranyl transferase or squalene synthesis at low micromolar concentrations (Lerner et al., 1995). GGTI-2133 blocks geranylgeranylation of target proteins via inhibition of geranylgeranyl transferase, while sparing farnesylation and squalene synthesis (Vasudevan et al., 1999).

While inhibition of squalene synthase and farnesyl transferase had only a mild effect on the growth of MDA-231 cells in 3D culture, inhibition of geranylgeranylation had a profound impact on both the growth and the invasive morphology of these cells in 3D culture (Figure 2.5A). To examine whether downregulation of geranylgeranylation is necessary for the phenotypic effects observed after mutant p53 depletion or HMG-CoA reductase inhibition, we performed add-back experiments using GGPP to cells either depleted of mutant p53 or cells treated with Simvastatin (Figure 2.5B and C, respectively). Since supplementation with geranylgeranyl pyrophosphate was sufficient to rescue the invasive phenotype in a portion of the population of MDA-231 cells in 3D culture, this suggests that geranylgeranylation is indeed a vital component of why breast cancers have selected for mutant p53 upregulation of the mevalonate pathway.

Mutant p53 is recruited to the promoters of genes encoding sterol biosynthesis enzymes

Much of the physiologic regulation of the sterol biosynthesis enzymes takes place at the transcriptional level in a manner that requires the sterol regulatory element binding proteins,

SREBP-1 and SREBP-2 (Sato, 2010). SREBPs activate the genes encoding nearly every key enzyme in both the fatty acid and sterol biosynthetic pathways (Horton et al., 2002). The SREBPs belong to the basic helix-loop-helix–leucine zipper (bHLH-Zip) family of transcription factors, but they are unique among this class of transcription factors in that they are synthesized as inactive precursors bound to the endoplasmic reticulum (ER), and only upon signals such as sterol depletion are they cleaved into mature fragments that translocate to the nucleus to bind to and regulate their target genes (Bengoechea-Alonso and Ericsson, 2007; Goldstein et al., 2006).

Most tumor-derived mutants of p53 are mutated in the DNA binding domain (DBD), and have thus lost the ability to recognize wild-type p53 response elements. Transcriptional regulation by mutant p53 may occur in many cases through interactions with other sequence-specific transcription factors binding to their cognate sites (Chicas et al., 2000; Di Agostino et al., 2006; Sampath et al., 2001; Stambolsky et al., 2010). Since the SREBPs are known to transactivate virtually every enzyme within the mevalonate pathway that we identified as being regulated by mutant p53 (in addition to many other sterol and fatty acid biosynthesis genes), and since mutant p53 requires a functional transactivation domain to disrupt morphogenesis in 3D culture (Figure 2.2), we hypothesized that mutant p53 might serve as a co-activator with one or more of the SREBPs. The following experiments support this hypothesis.

First, co-immunoprecipitation experiments using transiently expressed proteins demonstrated that mutant p53 (p53-R273H) can interact with the mature forms of all three family members, SREBP-1a, SREBP-1c and SREBP-2 (Figure 2.6A). Furthermore, we demonstrated that endogenous mutant p53 can interact with endogenous mature SREBP-2 in MDA-468 cells (Figure 2.6B) and MDA-231 cells (Figure 2.S6A). Second, mutant p53 regulates myriad SREBP target genes in addition to those identified by pathway analysis. MDA-468.shp53 cells were

grown in 3D culture with or without full levels of mutant p53 and the levels of a number of other SREBP target genes were assayed. In addition to the seven sterol biosynthesis genes uncovered by our initial pathway analysis, we validated ten additional sterol biosynthesis genes that are regulated by mutant p53 in MDA-468 cells (Figure 2.S6C), a subset of which are also regulated in MDA-231 cells (Figure 2.S6D). Of note, *DHCR24*, also known as Seladin1, was previously reported to be a transactivation target of another tumor-derived mutant, p53-R175H (Bossi et al., 2008). We also validated additional SREBP target genes that are not part of the mevalonate pathway as being regulated by mutant p53 in MDA-468 cells (Figure 2.S6E). Three of these genes (*FASN*, *ELOVL6* and *SCD*) encode key enzymes within the fatty acid biosynthesis pathway, suggesting that this pathway may also be upregulated by mutant p53. In addition, *INSIG1*, while not an enzyme in the sterol or fatty acid biosynthesis pathways, has been reported to be an obligate SREBP target gene (Goldstein et al., 2006), supporting the hypothesis that mutant p53 is cooperating with one of the SREBP proteins. Further, a comprehensive list of SREBP target genes (Reed et al., 2008) was queried to determine whether there was significant overlap with those identified by microarray analysis to be regulated by mutant p53 and there was a marked enrichment of SREBP target genes in the set of genes which were affected after mutant p53 depletion from breast cancer cells (Figure 2.S6B). Third, mutant p53 is recruited to the promoter regions of genes encoding sterol biosynthesis enzymes. Using quantitative chromatin immunoprecipitation (ChIP) analysis of MDA-468 cells with or without full levels of mutant p53, we identified significant binding by mutant p53 in the vicinity of sterol regulatory elements (SRE-1), the cognate binding sites for the SREBPs (Boone et al., 2009), in the promoter regions of all seven genes tested (Figure 2.6C and 6D). This ChIP signal varied between 2- to 4-fold greater than the signal at a negative region within the *CDKN1A* gene locus, and was consistently

reduced upon shRNA depletion of p53 (Figure 2.6C). We next used ChIP analysis to scan several regions upstream and downstream of the transcriptional start site (TSS) of the gene encoding HMG-CoA reductase (*HMGCR*). The region of peak binding by mutant p53 was located approximately 150 bp upstream of the TSS (Figure 2.6D), which corresponds to the known sterol regulatory element (Boone et al., 2009; Vallett et al., 1996). The peak signal, which was on the order of 3-fold greater than that seen with the negative region, was again significantly reduced in cells with depleted p53 (Figure 2.6D).

Fourth, SREBPs are likely necessary for the full recruitment of mutant p53 to these gene promoters. We downregulated by siRNA the SREBPs from MDA-468 cells and found that depletion of SREBP-1 slightly, but significantly, reduced the level of binding to the HMG-CoA reductase gene promoter (Figure 2.S6), while depletion of SREBP-2 substantially decreased the recruitment of mutant p53 to the HMG-CoA reductase gene promoter (Figure 2.6E). Additionally, Fatostatin, a recently described inhibitor of SREBP activation (Kamisuki et al., 2009), significantly reduced the level of mutant p53 binding to the HMG-CoA reductase gene promoter (Figure 2.6F). Finally, to examine whether SREBP activity affects the 3D morphology of breast cancer cells in 3D culture, we treated breast cancer cells with Fatostatin and indeed this inhibitor had a dramatic effect on the 3D morphology of MDA-231 cells (Figure 2.6G) and MDA-468 cells (data not shown). Taken together, our results strongly implicate a functional interaction with SREBPs as being critical for mutant p53-mediated upregulation of the mevalonate pathway genes.

TP53 **mutation correlates with elevated expression of sterol biosynthesis genes in human breast cancer patients**

To investigate whether the regulation of the mevalonate pathway by mutant p53 is generalizable to human patients, we examined five datasets consisting of a total of 812 human breast cancer patient samples (728 of these had known *TP53* mutational status). Each of these tumor specimens was previously subjected to both genome-wide expression analysis as well as sequencing of *TP53* (Enerly et al., 2011; Haakensen et al., 2010; Kyndi et al., 2009; Langerod et al., 2007; Miller et al., 2005; Muggerud et al., 2010; Myhre et al., 2010; Nielsen et al., 2006; Wiedswang et al., 2003). After stratifying patients based on the p53 mutational status of their tumors, we investigated the expression level of the sterol biosynthesis genes that we had previously identified as being regulated by mutant p53 in breast cancer cells grown in 3D culture. Remarkably, eleven of these sterol biosynthesis enzymes exhibited significantly higher expression levels in mutant p53 breast tumors compared to those bearing wild-type p53 across multiple datasets (Figure 2.7A, S7 and Table 2.S1).

We also performed the reciprocal analysis on these same breast cancer patient datasets, stratifying tumors based on their extent of expression of the mevalonate pathway genes and examining the mutation rate of *TP53*. Three main groups were observed from the hierarchical clustering of the expression matrix from 17 sterol biosynthesis genes on the 812 human breast cancer patient samples. Cluster I has the lowest sterol biosynthesis gene expression pattern and the lowest rate of *TP53* mutations (46/327 = 14.1%). Cluster III exhibits an intermediate expression level in these sterol biosynthesis genes and an intermediate rate of *TP53* mutations (94/272 = 34.6%). Cluster II has the highest expression pattern of sterol biosynthesis genes and exhibits the highest rate of *TP53* mutations (51/129 = 39.5%) (Figure 2.7B).

To test the biological significance of elevation of the mevalonate pathway in mutant p53 tumors, we examined whether upregulation of this pathway correlated with patient prognosis. It

is striking that cluster I, which has the lowest expression level of the mevalonate pathway genes, is correlated with a favorable prognosis, while cluster III, which has an intermediate expression pattern, correlates with an intermediate prognosis and cluster II, which has the highest expression of the mevalonate pathway genes, is associated with a significantly poorer survival probability (Figure 2.7C). Therefore, not only was elevation of the mevalonate pathway significantly correlated to a higher rate of p53 mutations, but these breast cancer patients also had a significantly decreased survival. We then examined each sterol biosynthesis gene individually to investigate which genes contribute most to the prognostic value. Elevated expression of nine out of the seventeen sterol biosynthesis genes correlated with significantly poorer prognosis in these breast cancer patients (Figure 2.7D).

Since breast cancer cells bearing mutant p53 appear to be particularly sensitive to inhibition of the mevalonate pathway in the 3D culture system, the fact that multiple members this pathway are upregulated in mutant p53 expressing human tumors and correlate with a poor prognosis may have important therapeutic implications.

DISCUSSION

Despite being one of the most well studied tumor suppressors, there is much evidence that once mutated, p53 can promote the progression of many cancers. With respect to breast cancer, tumor-derived mutants of p53 have been implicated in survival, chemoresistance, invasion, migration and metastasis (Adorno et al., 2009; Lim et al., 2009; Muller et al., 2006; Muller et al., 2009; Stambolsky et al., 2010). Since mammary tissue architecture is invariably disrupted during breast carcinogenesis, we sought to delineate the phenotypic effects of mutant

p53 in breast cancer. This study describes a possible oncogenic role for certain missense mutants of p53 in disrupting acinar morphogenesis of breast cells, explored using a three-dimensional culture system. Depletion of endogenous mutant p53 from breast cancer cells is sufficient to induce a phenotypic reversion in 3D culture and functional transactivation domains are necessary for mutant p53 to disrupt acinar morphogenesis.

We also demonstrate that mutant p53 upregulates seventeen genes encoding enzymes in the mevalonate pathway. The phenotypic effects following downregulation of mutant p53 can be recapitulated by inhibiting critical enzymes in the mevalonate pathway and can be reversed by supplementing breast cancer cells depleted of mutant p53 with two key intermediate metabolites produced by this pathway. Thus, flux through the mevalonate pathway is both necessary and sufficient for the phenotypic effects of mutant p53 on breast cancer cell morphogenesis in 3D culture. While it has been demonstrated previously that inhibition and/or downregulation of oncogenic signaling pathways can phenotypically revert breast cancer cells grown in 3D culture (Bissell et al., 2005; Wang et al., 1998; Weaver et al., 1997), we show here that downregulation of mutant p53 and/or inhibition of the mevalonate pathway can function in this capacity.

The mevalonate pathway has recently been implicated in multiple aspects of tumorigenesis, including proliferation, survival, invasion and metastasis (Clendening et al., 2010; Dimitroulakos et al., 1999; Kidera et al., 2010; Koyuturk et al., 2007; Wejde et al., 1992). Competitive inhibitors of the rate-limiting enzyme in the mevalonate pathway, HMG-CoA reductase, collectively known as statins, have been reported to be cancer-protective for certain malignancies, including breast cancer (Ahern et al., 2011; Blais et al., 2000; Cauley et al., 2003; Stein E A, 1993); however, the cancer-protective effects of statins are not without debate (Baigent et al., 2005; Browning and Martin, 2007). The statins have already been employed in

multiple preclinical models of breast cancer (Kubatka et al., 2011; Shibata et al., 2004) and it is noteworthy that at least two reports have already demonstrated a significant impact of Simvastatin treatment on growth of MDA-231 breast cancer xenografts in immunocompromised mice (Ghosh-Choudhury et al., 2010; Mori et al., 2009). In line with this, we were also able to demonstrate a significant impact of Simvastatin treatment on MDA-231 breast cancer cells *in vivo*.

It is interesting to note that at least one clinical study investigating the effect of statins in breast cancer noted a subgroup-specific protective effect: specifically, a significantly decreased incidence of hormone receptor-negative (ER-/PR-) tumors was documented in patients takings statins, while no such effect was observed for hormone receptor-positive tumors (Kumar et al., 2008). Preclinical models, employing either breast cancer cell lines or mouse models of breast cancer, also support a more dramatic role for statins in ER-/PR- breast cancers (Campbell et al., 2006; Garwood et al., 2010). Since the majority of breast tumors that bear p53 mutations most commonly are also immunohistochemically classified as ER-/PR- (Han et al., 2011; Sorlie et al., 2001), it is tempting to speculate that the observed anti-tumorigenic effects of statins are a consequence of mutant p53's upregulation of the mevalonate pathway.

Gene expression profiling of breast cancers has identified specific subtypes with important clinical, biologic and therapeutic implications (Perou et al., 2000). Using these expression signatures, most p53 mutations cluster in the basal-like subgroup of breast cancers, which has the poorest prognosis and is notoriously difficult to treat (Sorlie et al., 2001). Fascinatingly, using a combination of expression signatures and data from over 40,000 compounds screened in the NCI-60 cell lines, Mori et al. predicted three FDA-approved drugs to be most effective for treating basal-like breast cancers, two of which, Simvastatin and

Lovastatin, are inhibitors of HMG-CoA reductase (Mori et al., 2009). It will be exciting to examine whether stratifying breast cancer patients based on their p53 mutational status can resolve the apparent discrepancies within the rich body of literature linking statins and cancer.

Although we have implicated the mevalonate pathway in the phenotypic effects of mutant p53, it will be of great interest to further delineate the metabolite(s) as well as the downstream signaling pathways that are responsible for these phenotypic effects. While we have demonstrated that metabolic flux through the mevalonate pathway is sufficient to disrupt acinar morphogenesis, with a specific reliance on geranylgeranylation, we cannot rule out the possibility that one or more other metabolites are involved in the phenotypic effects that we observe in 3D culture. For example, metabolites such as mevalonic acid and farnesyl pyrophosphate have also been shown to promote cellular proliferation and/or cell survival (Fuchs et al., 2008; Graaf et al., 2004; Minutolo et al., 2005; Quesney-Huneeus et al., 1979; Riganti et al., 2011). Alternatively, cholesterol itself may promote survival pathways, by impinging upon the PI3K signaling pathway (Zhuang et al., 2002). If geranylgeranyl pyrophosphate is in fact the key metabolite, it will be very interesting to delineate the geranylgeranylated protein target(s) that mediate the oncogenic effects of the mevalonate pathway in breast cancer cells in 3D culture.

It is interesting to note that, in addition to the mevalonate pathway, a number of fatty acid biosynthesis genes were also significantly affected by mutant p53 depletion from breast cancer cells in 3D culture (Figure 2.3B and S6E). Intriguingly, this is the other major pathway controlled by the SREBP family of transcription factors (Horton et al., 2002). While much of our data points to a role for SREBP proteins in the regulation of the mevalonate pathway by mutant p53, a direct link is yet to be established. This regulation is likely to occur through one

or more of the SREBP proteins, but we cannot rule out the possibility that another factor or factors may also be involved. Mutant p53 may interact directly with elements in the promoters of the sterol biosynthesis genes or alternatively be recruited by a known mutant p53 interacting partner such as NF-Y, SP1, Ets-1 or VDR, which have been shown to recruit mutant p53 to their cognate binding sites (Chicas et al., 2000; Di Agostino et al., 2006; Sampath et al., 2001; Stambolsky et al., 2010).

A number of scenarios have been proposed to explain why human tumors select for mutations in p53 (Brosh and Rotter, 2009). First, mutant p53 may simply be selected for due to loss of wild-type p53 tumor suppressive activity. Second, mutant p53 may acquire neomorphic, or gain-of-function, activities which promote tumor growth, many of which have actually been shown to be diametrically opposed to those performed by wild-type p53 (Peart and Prives, 2006; Stambolsky et al., 2010). In line with this hypothesis are the findings that Stearoyl-CoA desaturase (encoded by *SCD*) is a repression target of wild-type p53 (Mirza et al., 2003; Riley et al., 2008) as well as that wild-type p53 can suppress a subset of SREBP target genes in a mouse model of obesity (Yahagi et al., 2003). As the pro-survival roles of wild-type p53 are becoming more apparent (Bensaad et al., 2006; Kim et al., 2009; Wu et al., 2005), a third scenario could be envisaged in which mutant p53 may retain and exaggerate certain wild-type p53 functions, while selectively losing certain tumor suppressive mechanisms such as the ability to induce cell cycle arrest and apoptosis. To examine the latter two hypotheses in relation to our findings that mutant p53 upregulates sterol biosynthesis genes, it will be interesting for future studies to examine whether wild-type p53 and/or its family members (p63 and p73) serve to repress sterol biosynthesis genes. Alternatively, the maintenance of high levels of sterol biosynthesis genes by mutant p53 may be a remnant of an unrecognized wild-type p53 function.

These speculations raise another important consideration, that not all p53 mutations are equivalent. Genetic alterations in p53 are often grouped into two classes based on the type of mutant p53 that they produce (Brosh and Rotter, 2009). Contact mutants, exemplified by p53-R273H, involve mutation of residues that are directly involved in protein-DNA contacts. Conformational mutants, typified by p53-R175H, result in conformational distortions in the p53 protein. Our findings that a subset of the sterol biosynthesis genes are significantly higher in large cohorts of human breast tumors bearing mutant p53 suggests that the ability of mutant p53 to upregulate the sterol biosynthesis genes is not constrained to a single class of mutations; however, it will be very interesting for follow-up studies to examine which tumor-derived mutants of p53 can regulate the levels of sterol biosynthesis genes.

In summary, our results demonstrate that mutant p53 can disrupt mammary acinar morphology and that downregulation of mutant p53 from malignant cells is sufficient to phenotypically revert these cells. Here we propose one mechanism, the upregulation of the mevalonate pathway, although one or more additional pathways may play a role. Specifically, we demonstrate that mutant p53 is recruited to the promoters of many sterol biosynthesis genes leading to their upregulation. We hypothesize that tumors bearing p53 mutations evolve to become highly reliant on metabolic flux through the mevalonate pathway, making them particularly sensitive to inhibition of this pathway. At a clinical level, inhibition of the mevalonate pathway, either alone or in combination with other therapies, may offer a novel, and much needed, therapeutic option for tumors bearing mutant p53.

EXPERIMENTAL PROCEDURES

Plasmids, siRNA, Antibodies and Reagents

pLNCX-Flag-p53-R175H, -G245S, -R248Q, -R248W, -R273H and -L22Q/W23S/W53Q/F54S-R175H, -G245S, -R248Q, -R248W, -R273H were generated from pLNCX-Flag-p53-WT using the Stratagene QuikChange Site-Directed Mutagenesis kit according to the manufacturer's instructions. Mutagenesis primer sequences are provided in Table 2.S2. pcDNA3.1-Myc-mSREBP-1a, -1c and -2 encode the mature forms of the SREBP transcription factors (Datta and Osborne, 2005). All constructs were verified by sequencing. p53 shRNA (2120) in STGM (tet-on) (Brekman et al., 2011) were used to establish cells with stable, inducible p53 knockdown. For transient knockdown experiments, siRNAs targeting SREBP1 (s129) or SREBP2 (s27) were purchased from Invitrogen. All-Stars (Control) and p53 siRNA were purchased from Qiagen.

p53 was detected using mAb 1801, DO-1 or 240. Anti-Actin (A2066), anti-Flag (F3165) and control IgG (I5381) antibodies were purchased from Sigma. Anti-Myc (sc-40) antibody was purchased from Santa Cruz. Anti-SREBP2 (1D2) is a monoclonal antibody raised against human SREBP-2 (hybridoma obtained from ATCC catalogue #CRL2545). Anti-SREBP2 (ab30682) antibody was purchased from Abcam. Alexa Fluor 594-Phalloidin (A12381) was purchased from Invitrogen.

Simvastatin (#10010344) and YM-53601 (#18113) were purchased from Cayman Chemicals. The following drugs were purchased from Sigma Aldrich: ALLN (A6185), Doxycycline (D9891), Simvastatin (S6196), Mevastatin (M2537), FTI-277 (F9803), GGTI-2133 (G5294), DL-Mevalolactone (M4667), DL-Mevalonic Acid 5-Phosphate (79849) and Geranylgeranyl Pyrophosphate (#G6025). Fatostatin was synthesized by the Medicinal Chemistry Core Facility

at the Sanford-Burnham Medical Research Institute as previously described (Kamisuki et al., 2009).

Cell Lines and Generation of Stable Cell Lines

MDA-468, MDA-231, HEK 293 and Phoenix cells were maintained in DMEM+10% FBS. MCF10A cells were maintained in DMEM/F12 supplemented with 5% horse serum, 10 μg/ml Insulin, 0.5 μg/ml Hydrocortisone and 20 ng/ml Epidermal Growth Factor (EGF). All cells were maintained at 37^{o}C in 5% CO_2.

To generate stable cell lines with inducible shRNA, constructs were introduced into MDA-231 or MDA-468 cells by the retroviral mediated gene transfer method. Briefly, Phoenix packaging cells were transfected by the calcium phosphate method with either an rtTA plasmid or a vector expressing p53 shRNA or no shRNA. The generated viruses were harvested and MDA-231 or MDA-468 cells were co-infected with the rtTA and one of the vectors. After selection with puromycin (vector with shRNA) and hygromycin (rtTA), clonal cell lines were generated by the limited dilution method. Clonal cell lines were selected based on the level of p53 knockdown. Experiments shown were carried out on clonal cell lines or stable pools (MDA-468.shp53 pool, MDA-468.shp53 clone 1F5 and MDA-231.shp53 clone 1D10). To induce shRNA expression, cells were treated with 8 μg/ml doxycycline (DOX) for time periods indicated in the figure legends.

To generate stable, mutant p53 expressing cells, MCF10A cells were infected with pLNCX-Flag-p53-R175H, -G245S, -R248Q, -R248W, or -R273H, or a transactivation-deficient version of each mutant (mTAD): pLNCX-Flag-p53-22/23/53/54-R175H, -G245S, -R248Q, -R248W, -R273H and selected in G418 to yield stable pools.

To generate shRNA-resistant mutant p53 expressing cells, MDA-231.shp53 (Clone 1D10) cells were infected with pLNCX or pLNCX-Flag-p53-R273H which lacks the target site for the p53 shRNA, found in the 3' UTR of the p53 mRNA. MDA-468.shp53 (Clone 1F5) cells were likewise infected with pLNCX, pLNCX-Flag-p53-R273H or pLNCX-Flag-p53-22/23/53/54-R273H (p53-R273H-mTAD) to generate shRNA-resistant mutant p53 expressing cells, either containing functional or non-functional transactivation domains, respectively. These cell lines were selected in G418 to generate stable pools.

Three Dimensional (3D) Culture

Three-dimensional culture was carried out as previously described (Debnath et al., 2003). Briefly, 8-well chamber slides were lined with 50 µl growth factor reduced Matrigel (BD Biosciences). Cells were then seeded at a density of 5,000 cells/well in Assay Medium (DMEM/F12 + 2% Horse Serum + 10 µg/ml Insulin + 0.5 µg/ml Hydrocortisone [+5 ng/ml EGF for MCF10A cultures]) containing 2% Matrigel. Cells were refed with Assay Medium containing 2% Matrigel every 4 days. For RNA/protein analysis from 3D cultures, 35 mm plates were lined with 500 µl Matrigel and cells were seeded at a density of 225,000 cells/plate in Assay Medium + 2% Matrigel. Cells were harvested using Cell Recovery Solution (BD Biosciences) according to the manufacturer's instructions.

Immunostaining and Microscopy

Cells were fixed using 2% formaldehyde at room temperature for at least 30 min. Cells were permeabilized for 10 min at 4^{o}C with 0.5% Triton X-100 and subsequently blocked for 1 hr at room temperature with PBS + 0.1% Tween-20 + 0.1% BSA + 10% goat serum. Primary antibodies were incubated with the cultures for 1-2 hr at room temperature, followed by washing,

and addition of fluorescently-conjugated secondary antibodies for 40 min at room temperature. Nuclei were counterstained with DRAQ5 (Cell Signaling #4084) or Propidium Iodide (Sigma #P4170). Confocal microscopy was conducted using an Olympus IX81 confocal microscope and analyzed using Fluoview software.

Microarray and Data analysis

RNA was isolated from three independent experiments of MDA-468.shp53 cells cultured under 3D conditions for 8 days in the presence or absence of DOX, reversed transcribed and hybridized to an Affymetrix GeneChip expression array (GEO Accession Number: GSE31812). Data was processed using the Robust Multichip Average (RMA) algorithm to give expression signals and paired t-test was applied for each probe. Probes with 1% significance were selected for Ingenuity Pathway Analysis.

For Gene Ontology (GO) analysis, probe sets with the Detection Above Background (DABG) p-value <0.05 in at least one sample were used. Gene expression was calculated based on the mean value of all the probesets for a gene. Gene expression changes were estimated by comparing cells grown in the presence of DOX versus those grown in the absence of DOX, and the three sample sets were analyzed separately. The GO annotation of genes was based on the NCBI Gene database. For each GO term, two p-values were calculated using Fisher's exact test by examining whether a significant fraction of genes associated with the GO term were up-regulated or down-regulated beyond the 1*standard deviation of all genes based on log2(ratio). The smaller p-value was used to represent the trend of regulation.

Quantitative RT-PCR

RNA was isolated from cells using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. Complementary DNA was transcribed using Qiagen Quantitect reverse transcription kit. Real-time PCR was carried out on an ABI StepOne Plus using SYBR green dye. Transcript levels were assayed in triplicate and normalized to RPL32 mRNA expression. Relative levels were calculated using the Comparative-Ct Method ($\Delta\Delta C_T$ method). All primers, unless otherwise noted, were designed with Primer Express (Applied Biosystems). qRT-PCR primer sequences are provided in Table 2.S2.

Drug Treatments

Simvastatin was activated by alkaline hydrolysis to the acidic form prior to usage as previously described (Sadeghi et al., 2000). Briefly, 5 mg of the Simvastatin pro-drug was dissolved in 0.125 ml of 95% ethanol, followed by 0.15 ml of 0.1 N NaOH and the solution was incubated at 50°C for 2 hr. The final solution was brought to a pH of ~7.2. Working solutions were stored in DMSO.

Cells in 3D culture were treated with Simvastatin or Mevastatin at the following concentrations: 100 nM or 1 μM. This range approximates clinically achievable serum concentrations in human patients (Dimitroulakos et al., 1999; Wong et al., 2002). Cells were treated with 6-Fluoromevalonate (200 μM) as previously described (Cuthbert and Lipsky, 1990). Cells were treated with YM-53601, FTI-277 or GGTI-2133 (Lerner et al., 1995; Ugawa et al., 2000; Vasudevan et al., 1999). Cells were treated with Fatostatin at either 2 μM or 20 μM as previously described (Kamisuki et al., 2009). Cells in 3D culture were treated on Day 1 or Day 4 of the 3D protocol (as described in the figure legends) and refed every 4 days with fresh drug.

Add-back experiments

MDA-468.shp53 or MDA-231.shp53 cells were cultured under 3D conditions in the presence (+DOX) or absence (-DOX) of doxycycline to deplete mutant p53. On Day 1 of 3D culture, cells cultured in the presence of doxycycline were supplemented with DL-Mevalolactone (1 mM)/DL-Mevalonic Acid 5-Phosphate (1 mM) or Geranygeranyl pyrophosphate (25 μM) and re-fed every 4 days.

MDA-468 or MDA-231 cells were pretreated with DL-Mevalolactone (1 mM)/DL-Mevalonic Acid 5-Phosphate (1 mM) or Geranylgeranyl pyrophosphate (25 μM) and then treated with Simvastatin (1 μM).

Soft-Agar Assay

35mm petri dishes were coated with base agar (0.6% in DME) and $2x10^4$ MDA-468 or $1x10^4$ MDA-231 cells were plated in top agar (0.3% in DME). Cultures were overlaid with 1.5 mL DME + 10% FBS with indicated drug concentrations. Drugs were changed every 3-4 days and culture media was changed every 7 days. Colonies were stained with 0.005% Crystal Violet and counted using a 4x objective on a phase contrast microscope.

Flow cytometry analysis

MDA-468 or MDA-231 cells were treated with indicated concentrations of Simvastatin for 24 hr and processed for FACS. Cell pellets were washed with phosphate buffered saline and fixed/permeabilized with 50% ice-cold ethanol. Pellets were washed and resuspended in 50 μg/ml ribonuclease A and 62.5 μg/ml propidium iodide. Samples were analyzed on the Becton Dickinson FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). The percentages of cells

in various phases of the cell cycle were quantified using the ModFit LT Version 3.0 program (Verity Software House, Topsham, ME, USA).

Mouse study

MDA-231 cells ($2x10^6$), resuspended in 50 µl media + 50 µl Matrigel were injected subcutaneously into 8 week-old female NOD-SCID mice. 14 days after implantation, tumors were measured by calipers and mice were paired by equal tumor volume and randomized to a Simvastatin or Control group (N=5 in both cases). Simvastatin group received Simvastatin (200 mg/kg/day) dissolved in H_2O + 1% carboxymethyl cellulose (w/v) and administered daily by oral gavage, Control group received the vehicle daily by oral gavage. Mice were weighed weekly and tumor measurements were performed weekly using a caliper. The volume of the tumor was calculated as $v = a^2*b$ (being a the small diameter and b the long diameter). After 21 days of treatment, mice were sacrificed and tumors were extracted and weighed.

Co-Immunoprecipitation of p53 and SREBP proteins

To detect exogenously expressed proteins, sub-confluent HEK 293 cells were transiently transfected with mutant p53 (Flag-p53-R273H) and an SREBP (Myc-mSREBP-1a, -1c or -2) expression constructs using Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection, cells were subjected to formaldehyde crosslinking (1% formaldehyde for 15 min), lysed in RIPA Buffer (150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 5mM EDTA, 50 mM Tris pH 8.0, 0.5 mM PMSF, protease inhibitors [1 µM benzamidine, 3 µg/ml leupeptin, 0.1 µg/ml bacitracin, and 1 µg/ml macroglobulin]) and sonicated. Anti-Flag antibody (4 µg) with protein A/G Sepharose beads (70 µl 1:1 slurry) was used to immunoprecipitate p53 from 2

mg whole cell lysate. Samples were then subjected to SDS-Page and immunoblotted with anti-Myc or anti-Flag antibodies.

To detect endogenously expressed proteins, MDA-468.shp53 cells were serum starved (2.5% serum) for 24 hours. Cells were harvested, re-suspended in hypotonic buffer (20 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, protease inhibitors [1 μM benzamidine, 3 μg/ml leupeptin, 0.1 μg/ml bacitracin, and 1 μg/ml macroglobulin]) for 10 min on ice, disrupted by passage through a 25-gauge needle and centrifuged at 1900 rpm for 10 min. The resultant nuclear pellet was suspended in lysis buffer (50 mM Tris-HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 0.5 mM PMSF, protease inhibitors [1 μM benzamidine, 3 μg/ml leupeptin, 0.1 μg/ml bacitracin, and 1 μg/ml macroglobulin]) and centrifuged at 13000 rpm for 20 min at 4°C. For co-immunoprecipitation of endogenous proteins the lysate was incubated at 4°C with either control IgG (Sigma, I5381) or anti-SREBP-2 antibody (1D2) for 6 hr and subsequently with protein A/G beads for 1 hr. The bead pellet was extensively washed in lysis buffer four times and then electrophoresed on 8% SDS-PAGE gels followed by immunoblotting using anti-SREBP-2 (1D2) and anti-p53 (DO-1) antibodies.

Quantitative Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) experiments were carried out as previously described (Beckerman et al., 2009). Briefly, MDA-468 cells were treated with 1% formaldehyde prior to lysis in RIPA Buffer and sonication to yield 500 bp fragments. Protein A/G Sepharose beads were conjugated to anti-p53 antibodies (1801/DO-1) which were subsequently used to immunoprecipitate p53 from 1 mg whole cell lysate. Quantitative ChIP was carried out on an ABI StepOne Plus using SYBR green dye. Genomic Locations of SRE-1 sites within the

promoters of sterol biosynthesis genes were located using a literature search: HMGCS1 (Inoue et al., 1998), HMGCR (Boone et al., 2009), MVK (Bishop et al., 1998), FDPS (Ishimoto et al., 2010), FDFT1 (Inoue et al., 1998), SQLE (Nagai et al., 2002) and CYP51A1 (Halder et al., 2002), respectively. ChIP primer sequences are provided in Table 2.S2.

Patient Data

Hierarchical clustering

Expression data for the sterol biosynthesis genes were extracted from individual cohorts (FW-MDG, MicMa, Ull, DBCG and Miller). Expression values per gene per dataset were standardized to have mean 0 and standard deviation 1 and further merged across the five datasets.

Unsupervised hierarchical clustering was used to discover groups based on the expression pattern of the sterol biosynthesis genes. In total, 17 sterol biosynthesis genes were used in the unsupervised hierarchical clustering.

Expression values of the 17 sterol biosynthesis genes on 812 samples (where rows indicate the identity of the genes, columns indicate the identity of the patients) were clustered using hierarchical clustering with Euclidean distance and ward linkage. Note that gene *MVD* was not present in the DBCG dataset; expression values of *MVD* on 615 samples were used for the distance calculation. The Kaplan-Meier survival curves were plotted for the resulting groups and the differences in clinical indications among the clusters were tested by a logrank test.

Univariate survival analysis for Sterol Biosynthesis Genes

Breast cancer specific death was used as survival endpoint for the analysis (n = 533 for *MVD* and

n = 723 for others). To remove batch effect across different cohorts, individual gene from each expression dataset was standardized to have mean 0 and standard deviation 1. Expression values per gene per cohort were pooled across the datasets. A univariate Cox proportional hazards model per sterol biosynthesis gene was then fitted:

$$h(t \mid \mathrm{X}) = h_0(t)\exp(\beta_1 \mathrm{X})$$

where **X** is the expression vector from the specific gene (variable), β_1 is the coefficient associated with a specific gene, and $h_0(t)$ is the (common) baseline hazard function.

The *Hazard Ratio* (HR) was used as an accuracy measure for the risk group prediction for categorical predictors. The larger the HR, the better is the discrimination between the groups of the patients, such as low- and high-risk. In our study, continuous covariates entering the Cox models were scaled into mean 0 with standard deviation 1. Thus the estimated HR on the standardized data characterized the relative risk for 1-standard-deviation increase in risk estimation by a specific sterol biosynthesis gene.

Benjamini Hochberg procedure (Benjamini and Hochberg, 1995) was used to adjust multiple comparisons across the tested genes (n = 17).

Expression of Sterol Biosynthesis Gene versus TP53 mutation status

One-tailed t test was performed to assess the significance of the increases in expression level for *TP53* mutated samples to those with wild type. The alternative hypothesis H_a was expression level of *TP53* mutated samples is higher than that of wild type samples.

For individual gene, the test was carried out on five breast cancer datasets: FW-MDG (Haakensen et al., 2010; Muggerud et al., 2010), MicMa (Enerly et al., 2011; Wiedswang et al.,

2003), ULL (Langerød et al., 2007), DBCG (Kyndi et al., 2009; Myhre et al., 2010; Nielsen et al., 2006) and Miller (Miller et al., 2005) respectively.

Combine p values

Since the datasets do not contain the same patients, we considered the conducted tests in each of the datasets for one gene were independent. An overall significance per gene across datasets was obtained using the Fisher's Omnibus (Fisher, 1932). Benjamini Hochberg procedure (Benjamini and Hochberg, 1995) was used to adjust multiple comparisons across the tested genes.

Fisher's method (Fisher, 1932) is used to combine the p values from several independent tests bearing upon the same overall hypothesis (H_0) into one test statistic F:

$$F_i = -2\sum_j \log(p_{ij})$$

where p_i is the p-value from the hypothesis testing for gene i in the jth dataset ($j = 1, \ldots k$; k is the total number of tests being combined; k was five in the study). When all the null hypotheses are true, test statistic F has a chi-square distribution with 2k degrees of freedom, Therefore, the corresponding overall p-value p_{0i} for one gene across all dataset was computed by:

$$p_{0i} = 1 - \chi^2(F_i, 2k)$$

Gene annotation mapping

The expression sets were annotated using Entrez gene identities. Genes of interest were mapped to each of the individual sets through Entrez gene IDs. For FW-MDG and MicMa set, the original Agilent probes were mapped to Entrez IDs using BioMart through R library biomaRt (Ensembl release 54/NCBI36 (hg18) human assembly). For Miller set, Affymetrix HG u133a

probes were mapped to Entrez IDs by BioMart under the same release. For ULL set, annotations for Stanford 43k cDNA array were retrieved from SMD SOURCE (http://smd.stanford.edu/cgi-bin/source/sourceSearch) under UniGene Build Number 222. Gene identity conversion on DBCG expression set was done using the provided chip annotation file for Applied Biosystems Human Genome Survey Microarray. For the probes shared the same Entrez gene identity, we selected probe(s) with the largest interquartile range (IQR: difference between the third and first quartiles) among the multiple hits. If this still left with more than one hit per Entrez ID, we further averaged the expression values of those probes for each sample.

Datasets

FW-MDG

Two expression sets FW (n = 109) (Muggerud et al., 2010) and MDG (n = 143) (Haakensen et al., 2010) were both from Agilent Whole Human Genome Oligo Microarrays 44k two color system. In addition, they both are early stage breast cancer cohorts and clinically similar. In this study, we merged the two datasets by gene-median centering on the original probe level. We also excluded normal samples in the MDG set in the study. In total, 139 breast tumors expression profiles with available information on *TP53* status entered the analysis. Among these, 28 samples with mutated *TP53* status and 111 samples with wild-type status.

MicMa

This cohort (Wiedswang et al., 2003) consists of mainly stage I and II breast cancers. mRNA expression profiling was performed on Agilent catalogue design whole human genome 4x44K one color oligo array. Among the 112 tumor samples with available *TP53* status in this sets, 39 samples with mutated *TP53* status and 73 samples with wild-type status.

ULL

This cohort consists of mainly stage I and II breast cancers. Eighty tumors, along with one normal breast tissue sample, were analyzed using Stanford cDNA 43k two color microarrays. We excluded the normal sample in the study, which left 80 tumor samples for the analysis. Among these, 20 samples with mutated *TP53* status and 60 samples with wild-type status.

DBCG

The DBCG series comprise a collection of tumor tissues from 3,083 high-risk Danish breast cancer patients diagnosed in the period 1982–1990 (Kyndi et al., 2009; Myhre et al., 2010; Nielsen et al., 2006). The profiling was carried out on the Applied Biosystems Human Genome Survey one color Microarray. For this study, there were 46 samples with mutated *TP53* status and 104 samples with wild-type status.

Miller

The Miller dataset (Miller et al., 2005) was downloaded from NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) with identifier GSE3494. Data were preprocessed and normalized as described previously (van Vliet et al., 2008). Among the 247 samples, there were 58 samples with mutated *TP53* status and 189 samples with wild type status.

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REFERENCES

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V., *et al.* (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell *137*, 87-98.

Ahern, T.P., Pedersen, L., Tarp, M., Cronin-Fenton, D.P., Garne, J.P., Silliman, R.A., Sorensen, H.T., and Lash, T.L. (2011). Statin Prescriptions and Breast Cancer Recurrence Risk: A Danish Nationwide Prospective Cohort Study. J Natl Cancer Inst.

Allred, D.C., Mohsin, S.K., and Fuqua, S.A. (2001). Histological and biological evolution of human premalignant breast disease. Endocr Relat Cancer *8*, 47-61.

Baigent, C., Keech, A., Kearney, P.M., Blackwell, L., Buck, G., Pollicino, C., Kirby, A., Sourjina, T., Peto, R., Collins, R., *et al.* (2005). Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet *366*, 1267-1278.

Beckerman, R., Donner, A.J., Mattia, M., Peart, M.J., Manley, J.L., Espinosa, J.M., and Prives, C. (2009). A role for Chk1 in blocking transcriptional elongation of p21 RNA during the S-phase checkpoint. Genes Dev *23*, 1364-1377.

Beliveau, A., Mott, J.D., Lo, A., Chen, E.I., Koller, A.A., Yaswen, P., Muschler, J., and Bissell, M.J. (2010). Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. Genes Dev *24*, 2800-2811.

Bengoechea-Alonso, M.T., and Ericsson, J. (2007). SREBP in signal transduction: cholesterol metabolism and beyond. Curr Opin Cell Biol *19*, 215-222.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological) *57*, 289-300.

Bensaad, K., Tsuruta, A., Selak, M.A., Vidal, M.N., Nakano, K., Bartrons, R., Gottlieb, E., and Vousden, K.H. (2006). TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell *126*, 107-120.

Bishop, R.W., Chambliss, K.L., Hoffmann, G.F., Tanaka, R.D., and Gibson, K.M. (1998). Characterization of the mevalonate kinase 5'-untranslated region provides evidence for coordinate regulation of cholesterol biosynthesis. Biochem Biophys Res Commun *242*, 518-524.

Bissell, M.J., Kenny, P.A., and Radisky, D.C. (2005). Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes. Cold Spring Harb Symp Quant Biol *70*, 343-356.

Bissell, M.J., Radisky, D.C., Rizki, A., Weaver, V.M., and Petersen, O.W. (2002). The organizing principle: microenvironmental influences in the normal and malignant breast. Differentiation *70*, 537-546.

Blais, L., Desgagne, A., and LeLorier, J. (2000). 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and the risk of cancer: a nested case-control study. Arch Intern Med *160*, 2363-2368.

Boone, L.R., Niesen, M.I., Jaroszeski, M., and Ness, G.C. (2009). In vivo identification of promoter elements and transcription factors mediating activation of hepatic HMG-CoA reductase by T3. Biochem Biophys Res Commun *385*, 466-471.

Bossi, G., Marampon, F., Maor-Aloni, R., Zani, B., Rotter, V., Oren, M., Strano, S., Blandino, G., and Sacchi, A. (2008). Conditional RNA interference in vivo to study mutant p53 oncogenic gain of function on tumor malignancy. Cell Cycle *7*, 1870-1879.

Brekman, A., Singh, K.E., Polotskaia, A., Kundu, N., and Bargonetti, J. (2011). A p53-independent role of Mdm2 in estrogen-mediated activation of breast cancer cell proliferation. Breast Cancer Res *13*, R3.

Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer *9*, 701-713.

Browning, D.R., and Martin, R.M. (2007). Statins and risk of cancer: a systematic review and metaanalysis. Int J Cancer *120*, 833-843.

Buchwald, H. (1992). Cholesterol inhibition, cancer, and chemotherapy. Lancet *339*, 1154-1156.

Campbell, M.J., Esserman, L.J., Zhou, Y., Shoemaker, M., Lobo, M., Borman, E., Baehner, F., Kumar, A.S., Adduci, K., Marx, C.*, et al.* (2006). Breast cancer growth prevention by statins. Cancer Res *66*, 8707-8714.

Cao, Z., Fan-Minogue, H., Bellovin, D.I., Yevtodiyenko, A., Arzeno, J., Yang, Q., Gambhir, S.S., and Felsher, D.W. (2011). MYC Phosphorylation, Activation, and Tumorigenic Potential in Hepatocellular Carcinoma Are Regulated by HMG-CoA Reductase. Cancer Res *71*, 2286-2297.

Casey, P.J., Solski, P.A., Der, C.J., and Buss, J.E. (1989). p21ras is modified by a farnesyl isoprenoid. Proc Natl Acad Sci U S A *86*, 8323-8327.

Cauley, J.A., Zmuda, J.M., Lui, L.Y., Hillier, T.A., Ness, R.B., Stone, K.L., Cummings, S.R., and Bauer, D.C. (2003). Lipid-lowering drug use and breast cancer in older women: a prospective study. J Womens Health (Larchmt) *12*, 749-756.

Chicas, A., Molina, P., and Bargonetti, J. (2000). Mutant p53 forms a complex with Sp1 on HIV-LTR DNA. Biochem Biophys Res Commun *279*, 383-390.

Clendening, J.W., Pandyra, A., Boutros, P.C., El Ghamrasni, S., Khosravi, F., Trentin, G.A., Martirosyan, A., Hakem, A., Hakem, R., Jurisica, I.*, et al.* (2010). Dysregulation of the mevalonate pathway promotes transformation. Proc Natl Acad Sci U S A *107*, 15051-15056.

Cuthbert, J.A., and Lipsky, P.E. (1990). Inhibition by 6-fluoromevalonate demonstrates that mevalonate or one of the mevalonate phosphates is necessary for lymphocyte proliferation. J Biol Chem *265*, 18568-18575.

Datta, S., and Osborne, T.F. (2005). Activation domains from both monomers contribute to transcriptional stimulation by sterol regulatory element-binding protein dimers. J Biol Chem *280*, 3338-3345.

Davidoff, A.M., Herndon, J.E., 2nd, Glover, N.S., Kerns, B.J., Pence, J.C., Iglehart, J.D., and Marks, J.R. (1991). Relation between p53 overexpression and established prognostic factors in breast cancer. Surgery *110*, 259-264.

Debnath, J., Mills, K.R., Collins, N.L., Reginato, M.J., Muthuswamy, S.K., and Brugge, J.S. (2002). The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell *111*, 29-40.

Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods *30*, 256-268.

Denoyelle, C., Vasse, M., Korner, M., Mishal, Z., Ganne, F., Vannier, J.P., Soria, J., and Soria, C. (2001). Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits the signaling pathways involved in the invasiveness and metastatic properties of highly invasive breast cancer cell lines: an in vitro study. Carcinogenesis *22*, 1139-1148.

Di Agostino, S., Strano, S., Emiliozzi, V., Zerbini, V., Mottolese, M., Sacchi, A., Blandino, G., and Piaggio, G. (2006). Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer Cell *10*, 191-202.

Dimitroulakos, J., Nohynek, D., Backway, K.L., Hedley, D.W., Yeger, H., Freedman, M.H., Minden, M.D., and Penn, L.Z. (1999). Increased sensitivity of acute myeloid leukemias to lovastatin-induced apoptosis: A potential therapeutic approach. Blood *93*, 1308-1318.

Enerly, E., Steinfeld, I., Kleivi, K., Leivonen, S.K., Aure, M.R., Russnes, H.G., Ronneberg, J.A., Johnsen, H., Navon, R., Rodland, E.*, et al.* (2011). miRNA-mRNA Integrated Analysis Reveals Roles for miRNAs in Primary Breast Tumors. PLoS One *6*, e16915.

Fisher, S.R.A. (1932). Statistical methods for research workers (Genesis Publishing Pvt Ltd).

Fried, L.F. (2008). Effects of HMG-CoA reductase inhibitors (statins) on progression of kidney disease. Kidney Int *74*, 571-576.

Friedrich, M.J. (2003). Studying cancer in 3 dimensions: 3-D models foster new insights into tumorigenesis. JAMA *290*, 1977-1979.

Fuchs, D., Berges, C., Opelz, G., Daniel, V., and Naujokat, C. (2008). HMG-CoA reductase inhibitor simvastatin overcomes bortezomib-induced apoptosis resistance by disrupting a geranylgeranyl pyrophosphate-dependent survival pathway. Biochem Biophys Res Commun *374*, 309-314.

Garwood, E.R., Kumar, A.S., Baehner, F.L., Moore, D.H., Au, A., Hylton, N., Flowers, C.I., Garber, J., Lesnikoski, B.A., Hwang, E.S., *et al.* (2010). Fluvastatin reduces proliferation and increases apoptosis in women with high grade breast cancer. Breast Cancer Res Treat *119*, 137-144.

Ghosh-Choudhury, N., Mandal, C.C., and Ghosh Choudhury, G. (2010). Simvastatin induces derepression of PTEN expression via NFkappaB to inhibit breast cancer cell growth. Cell Signal *22*, 749-758.

Goldstein, J.L., and Brown, M.S. (1990). Regulation of the mevalonate pathway. Nature *343*, 425-430.

Goldstein, J.L., DeBose-Boyd, R.A., and Brown, M.S. (2006). Protein sensors for membrane sterols. Cell *124*, 35-46.

Graaf, M.R., Richel, D.J., van Noorden, C.J., and Guchelaar, H.J. (2004). Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer. Cancer Treat Rev *30*, 609-641.

Haakensen, V.D., Biong, M., Lingjaerde, O.C., Holmen, M.M., Frantzen, J.O., Chen, Y., Navjord, D., Romundstad, L., Luders, T., Bukholm, I.K., *et al.* (2010). Expression levels of uridine 5'-diphospho-glucuronosyltransferase genes in breast tissue from healthy women are associated with mammographic density. Breast Cancer Res *12*, R65.

Halder, S.K., Fink, M., Waterman, M.R., and Rozman, D. (2002). A cAMP-responsive element binding site is essential for sterol regulation of the human lanosterol 14alpha-demethylase gene (CYP51). Mol Endocrinol *16*, 1853-1863.

Han, J.S., Cao, D., Molberg, K.H., Sarode, V.R., Rao, R., Sutton, L.M., and Peng, Y. (2011). Hormone Receptor Status Rather Than HER2 Status Is Significantly Associated With Increased Ki-67 and p53 Expression in Triple-Negative Breast Carcinomas, and High Expression of Ki-67 but Not p53 Is Significantly Associated With Axillary Nodal Metastasis in Triple-Negative and High-Grade Non-Triple-Negative Breast Carcinomas. Am J Clin Pathol *135*, 230-237.

Horton, J.D., Goldstein, J.L., and Brown, M.S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest *109*, 1125-1131.

Hussain, S.P., and Harris, C.C. (1998). Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. Cancer Res *58*, 4023-4037.

Inoue, J., Sato, R., and Maeda, M. (1998). Multiple DNA elements for sterol regulatory element-binding protein and NF-Y are responsible for sterol-regulated transcription of the genes for

human 3-hydroxy-3-methylglutaryl coenzyme A synthase and squalene synthase. J Biochem *123*, 1191-1198.

Ishimoto, K., Tachibana, K., Hanano, I., Yamasaki, D., Nakamura, H., Kawai, M., Urano, Y., Tanaka, T., Hamakubo, T., Sakai, J., *et al.* (2010). Sterol-regulatory-element-binding protein 2 and nuclear factor Y control human farnesyl diphosphate synthase expression and affect cell proliferation in hepatoblastoma cells. Biochem J *429*, 347-357.

Jakobisiak, M., Bruno, S., Skierski, J.S., and Darzynkiewicz, Z. (1991). Cell cycle-specific effects of lovastatin. Proc Natl Acad Sci U S A *88*, 3628-3632.

Kamisuki, S., Mao, Q., Abu-Elheiga, L., Gu, Z., Kugimiya, A., Kwon, Y., Shinohara, T., Kawazoe, Y., Sato, S., Asakura, K., *et al.* (2009). A small molecule that blocks fat synthesis by inhibiting the activation of SREBP. Chem Biol *16*, 882-892.

Katz, M.S., Minsky, B.D., Saltz, L.B., Riedel, E., Chessin, D.B., and Guillem, J.G. (2005). Association of statin use with a pathologic complete response to neoadjuvant chemoradiation for rectal cancer. Int J Radiat Oncol Biol Phys *62*, 1363-1370.

Kenny, P.A., Lee, G.Y., Myers, C.A., Neve, R.M., Semeiks, J.R., Spellman, P.T., Lorenz, K., Lee, E.H., Barcellos-Hoff, M.H., Petersen, O.W., *et al.* (2007). The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol *1*, 84-96.

Kidera, Y., Tsubaki, M., Yamazoe, Y., Shoji, K., Nakamura, H., Ogaki, M., Satou, T., Itoh, T., Isozaki, M., Kaneko, J., *et al.* (2010). Reduction of lung metastasis, cell invasion, and adhesion in mouse melanoma by statin-induced blockade of the Rho/Rho-associated coiled-coil-containing protein kinase pathway. J Exp Clin Cancer Res *29*, 127.

Kim, E., Giese, A., and Deppert, W. (2009). Wild-type p53 in cancer cells: when a guardian turns into a blackguard. Biochem Pharmacol *77*, 11-20.

Koyuturk, M., Ersoz, M., and Altiok, N. (2007). Simvastatin induces apoptosis in human breast cancer cells: p53 and estrogen receptor independent pathway requiring signalling through JNK. Cancer Lett *250*, 220-228.

Kubatka, P., Zihlavnikova, K., Kajo, K., Pec, M., Stollarova, N., Bojkova, B., Kassayova, M., and Orendas, P. (2011). Antineoplastic effects of simvastatin in experimental breast cancer. Klin Onkol *24*, 41-45.

Kumar, A.S., Benz, C.C., Shim, V., Minami, C.A., Moore, D.H., and Esserman, L.J. (2008). Estrogen receptor-negative breast cancer is less likely to arise among lipophilic statin users. Cancer Epidemiol Biomarkers Prev *17*, 1028-1033.

Kyndi, M., Overgaard, M., Nielsen, H.M., Sorensen, F.B., Knudsen, H., and Overgaard, J. (2009). High local recurrence risk is not associated with large survival reduction after postmastectomy radiotherapy in high-risk breast cancer: a subgroup analysis of DBCG 82 b&c. Radiother Oncol *90*, 74-79.

Lang, G.A., Iwakuma, T., Suh, Y.A., Liu, G., Rao, V.A., Parant, J.M., Valentin-Vega, Y.A., Terzian, T., Caldwell, L.C., Strong, L.C.*, et al.* (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell *119*, 861-872.

Langan, T.J., and Volpe, J.J. (1986). Obligatory relationship between the sterol biosynthetic pathway and DNA synthesis and cellular proliferation in glial primary cultures. J Neurochem *46*, 1283-1291.

Langerød, A., Zhao, H., Borgan, O., Nesland, J., Bukholm, I., Ikdahl, T., Karesen, R., Borresen-Dale, A., and Jeffrey, S. (2007). TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. Breast Cancer Res *9*, R30.

Langerod, A., Zhao, H., Borgan, O., Nesland, J.M., Bukholm, I.R., Ikdahl, T., Karesen, R., Borresen-Dale, A.L., and Jeffrey, S.S. (2007). TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. Breast Cancer Res *9*, R30.

Laptenko, O., and Prives, C. (2006). Transcriptional regulation by p53: one protein, many possibilities. Cell Death Differ *13*, 951-961.

Larsson, O. (1996). HMG-CoA reductase inhibitors: role in normal and malignant cells. Crit Rev Oncol Hematol *22*, 197-212.

Lerner, E.C., Qian, Y., Blaskovich, M.A., Fossum, R.D., Vogt, A., Sun, J., Cox, A.D., Der, C.J., Hamilton, A.D., and Sebti, S.M. (1995). Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic Ras signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. J Biol Chem *270*, 26802-26806.

Lim, L.Y., Vidnovic, N., Ellisen, L.W., and Leong, C.O. (2009). Mutant p53 mediates survival of breast cancer cells. Br J Cancer *101*, 1606-1612.

Lin, J., Chen, J., Elenbaas, B., and Levine, A.J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes Dev *8*, 1235-1246.

Lin, J., Teresky, A.K., and Levine, A.J. (1995). Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants. Oncogene *10*, 2387-2390.

Martin, K.J., Patrick, D.R., Bissell, M.J., and Fournier, M.V. (2008). Prognostic breast cancer signature identified from 3D culture model accurately predicts clinical outcome across independent datasets. PLoS One *3*, e2994.

Matas, D., Sigal, A., Stambolsky, P., Milyavsky, M., Weisz, L., Schwartz, D., Goldfinger, N., and Rotter, V. (2001). Integrity of the N-terminal transcription domain of p53 is required for mutant p53 interference with drug-induced apoptosis. EMBO J *20*, 4163-4172.

Miller, L.D., Smeds, J., George, J., Vega, V.B., Vergara, L., Ploner, A., Pawitan, Y., Hall, P., Klaar, S., Liu, E.T.*, et al.* (2005). An expression signature for p53 status in human breast cancer

predicts mutation status, transcriptional effects, and patient survival. Proc Natl Acad Sci U S A *102*, 13550-13555.

Minutolo, F., Asso, V., Bertini, S., Betti, L., Ciriaco, M., Danesi, R., Gervasi, G., Ghilardi, E., Giovanetti, E., Giannaccini, G., *et al.* (2005). Variously substituted (phosphonoacetamido)oxy analogues of geranylgeranyl diphosphate (GGdP) as GGdP-transferase (GGTase) inhibitors and antiproliferative agents. Med Chem *1*, 239-244.

Mirza, A., Wu, Q., Wang, L., McClanahan, T., Bishop, W.R., Gheyas, F., Ding, W., Hutchins, B., Hockenberry, T., Kirschmeier, P., *et al.* (2003). Global transcriptional program of p53 target genes during the process of apoptosis and cell cycle progression. Oncogene *22*, 3645-3654.

Mori, S., Chang, J.T., Andrechek, E.R., Potti, A., and Nevins, J.R. (2009). Utilization of genomic signatures to identify phenotype-specific drugs. PLoS One *4*, e6772.

Muggerud, A.A., Hallett, M., Johnsen, H., Kleivi, K., Zhou, W., Tahmasebpoor, S., Amini, R.M., Botling, J., Borresen-Dale, A.L., Sorlie, T., *et al.* (2010). Molecular diversity in ductal carcinoma in situ (DCIS) and early invasive breast cancer. Mol Oncol *4*, 357-368.

Muller, M., Schleithoff, E.S., Stremmel, W., Melino, G., Krammer, P.H., and Schilling, T. (2006). One, two, three--p53, p63, p73 and chemosensitivity. Drug Resist Updat *9*, 288-306.

Muller, P.A., Caswell, P.T., Doyle, B., Iwanicki, M.P., Tan, E.H., Karim, S., Lukashchuk, N., Gillespie, D.A., Ludwig, R.L., Gosselin, P., *et al.* (2009). Mutant p53 drives invasion by promoting integrin recycling. Cell *139*, 1327-1341.

Muthuswamy, S.K., Li, D., Lelievre, S., Bissell, M.J., and Brugge, J.S. (2001). ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol *3*, 785-792.

Myhre, S., Mohammed, H., Tramm, T., Alsner, J., Finak, G., Park, M., Overgaard, J., Borresen-Dale, A.L., Frigessi, A., and Sorlie, T. (2010). In silico ascription of gene expression differences to tumor and stromal cells in a model to study impact on breast cancer outcome. PLoS One *5*, e14002.

Nagai, M., Sakakibara, J., Nakamura, Y., Gejyo, F., and Ono, T. (2002). SREBP-2 and NF-Y are involved in the transcriptional regulation of squalene epoxidase. Biochem Biophys Res Commun *295*, 74-80.

Nielsen, H.M., Overgaard, M., Grau, C., Jensen, A.R., and Overgaard, J. (2006). Study of failure pattern among high-risk breast cancer patients with or without postmastectomy radiotherapy in addition to adjuvant systemic therapy: long-term results from the Danish Breast Cancer Cooperative Group DBCG 82 b and c randomized studies. J Clin Oncol *24*, 2268-2275.

Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell *119*, 847-860.

Olivier, M., Langerod, A., Carrieri, P., Bergh, J., Klaar, S., Eyfjord, J., Theillet, C., Rodriguez, C., Lidereau, R., Bieche, I., *et al.* (2006). The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. Clin Cancer Res *12*, 1157-1167.

Peart, M.J., and Prives, C. (2006). Mutant p53 gain of function: the NF-Y connection. Cancer Cell *10*, 173-174.

Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., *et al.* (2000). Molecular portraits of human breast tumours. Nature *406*, 747-752.

Petersen, O.W., Ronnov-Jessen, L., Howlett, A.R., and Bissell, M.J. (1992). Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc Natl Acad Sci U S A *89*, 9064-9068.

Petitjean, A., Achatz, M.I., Borresen-Dale, A.L., Hainaut, P., and Olivier, M. (2007). TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene *26*, 2157-2165.

Quesney-Huneeus, V., Wiley, M.H., and Siperstein, M.D. (1979). Essential role for mevalonate synthesis in DNA replication. Proc Natl Acad Sci U S A *76*, 5056-5060.

Reed, B.D., Charos, A.E., Szekely, A.M., Weissman, S.M., and Snyder, M. (2008). Genome-wide occupancy of SREBP1 and its partners NFY and SP1 reveals novel functional roles and combinatorial regulation of distinct classes of genes. PLoS Genet *4*, e1000133.

Riganti, C., Pinto, H., Bolli, E., Belisario, D.C., Calogero, R.A., Bosia, A., and Cavallo, F. (2011). Atorvastatin modulates anti-proliferative and pro-proliferative signals in Her2/neu-positive mammary cancer. Biochem Pharmacol.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol *9*, 402-412.

Rotter, V. (1983). p53, a transformation-related cellular-encoded protein, can be used as a biochemical marker for the detection of primary mouse tumor cells. Proc Natl Acad Sci U S A *80*, 2613-2617.

Sadeghi, M.M., Collinge, M., Pardi, R., and Bender, J.R. (2000). Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. J Immunol *165*, 2712-2718.

Sampath, J., Sun, D., Kidd, V.J., Grenet, J., Gandhi, A., Shapiro, L.H., Wang, Q., Zambetti, G.P., and Schuetz, J.D. (2001). Mutant p53 cooperates with ETS and selectively up-regulates human MDR1 not MRP1. J Biol Chem *276*, 39359-39367.

Sanchez, C.A., Rodriguez, E., Varela, E., Zapata, E., Paez, A., Masso, F.A., Montano, L.F., and Loopez-Marure, R. (2008). Statin-induced inhibition of MCF-7 breast cancer cell proliferation is

related to cell cycle arrest and apoptotic and necrotic cell death mediated by an enhanced oxidative stress. Cancer Invest *26*, 698-707.

Sato, R. (2010). Sterol metabolism and SREBP activation. Arch Biochem Biophys *501*, 177-181.

Schafer, Z.T., Grassian, A.R., Song, L., Jiang, Z., Gerhart-Hines, Z., Irie, H.Y., Gao, S., Puigserver, P., and Brugge, J.S. (2009). Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. Nature *461*, 109-113.

Shachaf, C.M., Perez, O.D., Youssef, S., Fan, A.C., Elchuri, S., Goldstein, M.J., Shirer, A.E., Sharpe, O., Chen, J., Mitchell, D.J., *et al.* (2007). Inhibition of HMGcoA reductase by atorvastatin prevents and reverses MYC-induced lymphomagenesis. Blood *110*, 2674-2684.

Shibata, M.A., Ito, Y., Morimoto, J., and Otsuki, Y. (2004). Lovastatin inhibits tumor growth and lung metastasis in mouse mammary carcinoma model: a p53-independent mitochondrial-mediated apoptotic mechanism. Carcinogenesis *25*, 1887-1898.

Shibata, M.A., Kavanaugh, C., Shibata, E., Abe, H., Nguyen, P., Otsuki, Y., Trepel, J.B., and Green, J.E. (2003). Comparative effects of lovastatin on mammary and prostate oncogenesis in transgenic mouse models. Carcinogenesis *24*, 453-459.

Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., *et al.* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A *98*, 10869-10874.

Soussi, T., and Lozano, G. (2005). p53 mutation heterogeneity in cancer. Biochem Biophys Res Commun *331*, 834-842.

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., Shiff, I., Kogan, I., Shay, M., Kalo, E., *et al.* (2010). Modulation of the vitamin D3 response by cancer-associated mutant p53. Cancer Cell *17*, 273-285.

Stein E A, L.P., Steiner P. (1993). Lovastatin 5-year safety and efficacy study. Lovastatin Study Groups I through IV. Arch Intern Med *153*, 1079-1087.

Ugawa, T., Kakuta, H., Moritani, H., Matsuda, K., Ishihara, T., Yamaguchi, M., Naganuma, S., Iizumi, Y., and Shikama, H. (2000). YM-53601, a novel squalene synthase inhibitor, reduces plasma cholesterol and triglyceride levels in several animal species. Br J Pharmacol *131*, 63-70.

Unger, T., Mietz, J.A., Scheffner, M., Yee, C.L., and Howley, P.M. (1993). Functional domains of wild-type and mutant p53 proteins involved in transcriptional regulation, transdominant inhibition, and transformation suppression. Mol Cell Biol *13*, 5186-5194.

Vallett, S.M., Sanchez, H.B., Rosenfeld, J.M., and Osborne, T.F. (1996). A direct role for sterol regulatory element binding protein in activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene. J Biol Chem *271*, 12247-12253.

van Vliet, M.H., Reyal, F., Horlings, H.M., van de Vijver, M.J., Reinders, M.J., and Wessels, L.F. (2008). Pooling breast cancer datasets has a synergetic effect on classification performance and improves signature stability. BMC Genomics *9*, 375.

Vargo-Gogola, T., and Rosen, J.M. (2007). Modelling breast cancer: one size does not fit all. Nat Rev Cancer *7*, 659-672.

Vasudevan, A., Qian, Y., Vogt, A., Blaskovich, M.A., Ohkanda, J., Sebti, S.M., and Hamilton, A.D. (1999). Potent, highly selective, and non-thiol inhibitors of protein geranylgeranyltransferase-I. J Med Chem *42*, 1333-1340.

Venot, C., Maratrat, M., Sierra, V., Conseiller, E., and Debussche, L. (1999). Definition of a p53 transactivation function-deficient mutant and characterization of two independent p53 transactivation subdomains. Oncogene *18*, 2405-2410.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Wang, F., Weaver, V.M., Petersen, O.W., Larabell, C.A., Dedhar, S., Briand, P., Lupu, R., and Bissell, M.J. (1998). Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc Natl Acad Sci U S A *95*, 14821-14826.

Weaver, V.M., Petersen, O.W., Wang, F., Larabell, C.A., Briand, P., Damsky, C., and Bissell, M.J. (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol *137*, 231-245.

Wejde, J., Blegen, H., and Larsson, O. (1992). Requirement for mevalonate in the control of proliferation of human breast cancer cells. Anticancer Res *12*, 317-324.

Wiedswang, G., Borgen, E., Karesen, R., Kvalheim, G., Nesland, J.M., Qvist, H., Schlichting, E., Sauer, T., Janbu, J., Harbitz, T.*, et al.* (2003). Detection of isolated tumor cells in bone marrow is an independent prognostic factor in breast cancer. J Clin Oncol *21*, 3469-3478.

Wong, W.W., Dimitroulakos, J., Minden, M.D., and Penn, L.Z. (2002). HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia *16*, 508-519.

Wrobel, C.N., Debnath, J., Lin, E., Beausoleil, S., Roussel, M.F., and Brugge, J.S. (2004). Autocrine CSF-1R activation promotes Src-dependent disruption of mammary epithelial architecture. J Cell Biol *165*, 263-273.

Wu, W.S., Heinrichs, S., Xu, D., Garrison, S.P., Zambetti, G.P., Adams, J.M., and Look, A.T. (2005). Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma. Cell *123*, 641-653.

Yahagi, N., Shimano, H., Matsuzaka, T., Najima, Y., Sekiya, M., Nakagawa, Y., Ide, T., Tomita, S., Okazaki, H., Tamura, Y.*, et al.* (2003). p53 Activation in adipocytes of obese mice. J Biol Chem *278*, 25395-25400.

Yan, W., and Chen, X. (2010). Characterization of functional domains necessary for mutant p53 gain of function. J Biol Chem *285*, 14229-14238.

Yoshida, Y., Kawata, M., Katayama, M., Horiuchi, H., Kita, Y., and Takai, Y. (1991). A geranylgeranyltransferase for rhoA p21 distinct from the farnesyltransferase for ras p21S. Biochem Biophys Res Commun *175*, 720-728.

Zhan, L., Rosenberg, A., Bergami, K.C., Yu, M., Xuan, Z., Jaffe, A.B., Allred, C., and Muthuswamy, S.K. (2008). Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. Cell *135*, 865-878.

Zhang, Y., Yan, W., and Chen, X. (2011). Mutant p53 disrupts MCF-10A cell polarity in 3-dimensional culture via epithelial-to-mesenchymal transitions. J Biol Chem.

Zhuang, L., Lin, J., Lu, M.L., Solomon, K.R., and Freeman, M.R. (2002). Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. Cancer Res *62*, 2227-2231.

FIGURE LEGENDS

Figure 2.1 Depletion of mutant p53 from breast cancer cells induces a phenotypic reversion in 3D culture

(A) Depletion of mutant p53 dramatically affects 3D morphology of MDA-231 cells. MDA-231.shp53 cells were grown under 3D conditions for 8 days in the absence of DOX, thus retaining full levels of mutant p53, or grown in the presence of DOX to knockdown endogenous mutant p53. Representative differential interference contrast (DIC) images are shown. Scale Bar, 200 μm.

(B) shRNA mediated reduction of mutant p53 in MDA-231 cells. MDA-231.shp53 cells were grown in 3D culture for 8 days in the presence or absence of DOX as indicated prior to lysis and immunoblotting analysis as in Methods. p53 was detected using anti-p53 antibody (PAb1801). Actin serves as a loading control.

(C) Morphologic categories in MDA-468 cells. MDA-468.shp53 cells were grown in 3D cultures for 8 days and structures were grouped into three morphological categories: Malignant, Intermediate and Hollow Lumen. Actin cytoskeleton was stained with Phalloidin (Green) and nuclei were stained with DRAQ5 (Red). Structures were analyzed by confocal microscopy. Scale bar, 50 μm.

(D) Depletion of mutant p53 induces a phenotypic reversion in MDA-468 cells. MDA-468.shp53 cells were grown in 3D cultures for 8 days in the presence of DOX, leading to induction of an shRNA targeting p53, and thus to depleted levels of mutant p53. Left panel:

GFP (Green) serves as a marker for shRNA induction. Right panel: Nuclei were stained with DRAQ5 (Red) and analyzed by confocal microscopy. The larger structure is representative of intermediate colony morphologies, while the smaller structure is representative of acinus-like structures with hollow lumen morphology. White arrow indicates cell debris from apoptosis within the luminal space. Scale bar, 50 μm.

(E) Knockdown of mutant p53 in MDA-468 cells. MDA-468.shp53 cells were grown in 3D culture for 8 days in the presence or absence of DOX as indicated and processed as in (B). p53 was detected using an anti-p53 antibody (PAb1801). Actin serves as a loading control.

(F) Morphometry of MDA-468.shp53 pooled population. A stable pool of MDA-468.shp53 cells were grown in 3D cultures for 8 days in the presence or absence of DOX as indicated and structures were analyzed by confocal microscopy and categorized as in (C). Left panel illustrates population distribution. Right panel shows the percent of acinus-like structures with hollow lumens without (-) or with (+) mutant p53 depletion by treatment with DOX. Structures (50-100) were counted for each condition. *denotes $p<0.01$.

(G) Morphometry of MDA-468.shp53 clonal population. A stable clone of MDA-468.shp53 cells were grown in 3D cultures for 8 days in the presence or absence of DOX as indicated and structures were analyzed by confocal microscopy as in (F). Structures (50-100) were counted for each condition and plotted as a percentage of the population. An average of two experiments is shown.

Figure 2.2 Mutant p53 requires functional transactivation sub-domains to disrupt morphology of mammary cells in 3D culture

(A) MDA-468.shp53 cells expressing a control vector (pLNCX) were grown in 3D cultures for 5 days in the absence of DOX thus retaining full levels of mutant p53 (left panel), or grown in the presence of DOX inducing an shRNA that targets p53 (right panel), leading to depleted levels of mutant p53 as in Figure 2.1 or in (D) below. Representative DIC images are shown. Scale bar, 200 μm.

(B) MDA-468.shp53 cells expressing an shRNA-resistant Flag-tagged p53-R273H were grown in 3D cultures for 5 days in the absence or presence of DOX as in (A). Representative DIC images are shown. Scale bar, 200 μm.

(C) MDA-468.shp53 cells expressing an shRNA-resistant Flag-tagged p53-R273H-mTAD (mutant p53 with non-functional transactivation region, p53-R273H- L22Q/W23S/W53Q/F54S) were grown in 3D cultures for 5 days in the absence or presence of DOX as in (A). Representative DIC images are shown. Scale bar, 200 μm.

(D) Immunoblot of mutant p53 in MDA-468 cells. Cells either with control vector or expressing shRNA resistant versions of Flag-tagged p53-R273H mutant p53 or transactivation defective Flag-tagged p53-R273-mTAD were grown in 3D culture for 5 days in the absence or presence of DOX as indicated followed by lysis and processing for immunoblotting as in Figure 2.1B. p53 was detected using an anti-p53 antibody (PAb240). Note that exogenously expressed Flag-

tagged mutant p53 variants migrate more slowly than endogenously expressed mutant p53. Actin serves as a loading control.

Figure 2.3 Knockdown of mutant p53 from breast cancer cells in 3D culture significantly downregulates the mevalonate pathway

(A) Pathway analysis of breast cancer cells following mutant p53 depletion. Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). Significant ($p<0.01$) expression changes from genome-wide expression analysis were queried. Blue bars that cross the threshold line ($p<0.05$) represent pathways that are significantly changed following mutant p53 depletion from MDA-468 cells.

(B) Biological processes significantly altered by mutant p53 in 3D culture. Significant expression changes from genome-wide expression analysis were analyzed by Gene Ontology (GO) analysis. 1, 2, 3 represent three independent experiments. GO terms were sorted based their significance and redundant terms were discarded.

(C) Validation of sterol biosynthesis genes regulated by mutant p53. MDA-468.shp53 cells were grown in 3D cultures for 8 days in the presence or absence of DOX as indicated to deplete cells of mutant p53. Isolated RNA was reverse transcribed and qRT-PCR was performed for the seven sterol biosynthesis genes identified by Ingenuity Pathway Analysis: *HMGCR*, HMG-CoA reductase; *MVK*, Mevalonate Kinase; *MVD*, Mevalonate Decarboxylase; *FDPS*, Farnesyl Diphosphate Synthase; *SQLE*, Squalene Epoxidase; *LSS*, Lanosterol Synthase; *DHCR7*, 7-

Dehydrocholesterol reductase. Data is presented as mean ± st dev of three independent experiments. **indicates $p<0.005$ by two-sided t-test.

Figure 2.4 Downregulation of the mevalonate pathway is both necessary and sufficient to phenotypically revert breast cancer cells in 3D culture

(A) Intermediate metabolites rescue the phenotypic effects of depleting breast cancer cells of mutant p53. MDA-468.shp53 cells were grown in 3D cultures for 8 days in the presence or absence of DOX to deplete mutant p53. Parallel wells of cells which were grown in the presence of DOX were supplemented with metabolites produced within the mevalonate pathway: mevalonic acid/mevalonic acid-phosphate (MVA/MVAP) beginning on Day 1. Morphological categories as indicated were determined for 50-100 structures using confocal microscopy which were then plotted as a percentage of the population. A representative experiment is shown here and a second representative experiment is shown in Figure 2.S4.

(B) Inhibition of the mevalonate pathway affects MDA-468 cell morphology in 3D cultures. MDA-468 cells were grown in 3D culture conditions for 13 days untreated or treated with vehicle (DMSO), Simvastatin (100 nM) or Simvastatin (1 μM) as indicated. Drugs were added on Day 4. Scale Bar, 200 μm.

(C) Inhibition of the mevalonate pathway affects MDA-231 cell morphology in 3D cultures. MDA-231 cells were grown in 3D culture conditions for 13 days untreated or treated with vehicle (DMSO), Simvastatin (100 nM) or Simvastatin (1 μM) as indicated. Drugs were added on Day 4. Scale Bar, 200 μm.

(D) The effects of Simvastatin are due to inhibition of HMG-CoA reductase. MDA-468 cells (top panel) or MDA-231 cells (bottom panel) were grown in 3D cultures for 13 days with Simvastatin (1 μM) as in (B) and (C), respectively, but were supplemented with mevalonic acid/mevalonic acid-phosphate, the early enzymatic products after HMG-CoA reductase. Scale Bar, 200 μm.

(E) Supplementation with mevalonic acid is sufficient to block luminal clearance in MCF10A cells. MCF10A cells were grown in 3D culture for 8 days in the absence (Control) or presence (MVA) of 1 mM mevalonic acid. Nuclei were stained with DRAQ5 (Red) and structures were analyzed by confocal microscopy for the presence of a hollow or filled lumen (right panel). Structures (50-100) were counted for each condition. An average of two experiments is presented. Scale Bar, 50 μm.

Figure 2.5 Modulation of geranylgeranylation mediates many of the phenotypic effects of mutant p53 depletion and HMG-CoA reductase inhibition in MDA-231 cells

(A) Inhibition of downstream enzymes in the mevalonate pathway affects MDA-231 cell morphology in 3D cultures. MDA-231 cells were grown in 3D culture conditions for 8 days untreated or treated with vehicle (DMSO), YM-53601 (1 μM), FTI-277 (1 μM) or GGTI-2133 (1 μM) as indicated. Drugs were added on Day 1. Scale Bar, 200 μm.

(B) Geranylgeranyl pyrophosphate can partially rescue the morphological effects of mutant p53 depletion. MDA-231.shp53 cells were grown in 3D culture conditions for 9 days in the absence

(-DOX) or presence (+DOX) of doxycycline as indicated. Parallel wells of cells which were grown in the presence of DOX were supplemented with geranylgeranyl pyrophosphate (GGPP) (25 μM) beginning on Day 1. Scale Bar, 200 μm.

(C) Geranylgeranyl pyrophosphate can rescue the morphological effects of HMG-CoA reductase inhibition. MDA-231 cells were grown in 3D culture conditions for 13 days either treated with vehicle (DMSO) or Simvastatin (1 μM) as indicated. Parallel wells of cells which were grown in the presence of Simvastatin (1 μM) were supplemented with geranylgeranyl pyrophosphate (GGPP) (25 μM) beginning on Day 1. Scale Bar, 200 μm.

Figure 2.6 Mutant p53 is recruited to mevalonate pathway gene promoters and this recruitment is dependent on SREBP proteins

(A) Mutant p53 interacts with sterol regulatory element binding proteins. HEK 293 cells were transiently transfected with Flag-p53-R273H and either Myc-mSREBP-1a, -1c or -2. Cells were subjected to crosslinking with formaldehyde prior to cell lysis and sonication as described in Methods. Mutant p53 was immunoprecipitated from 2 mg whole cell lysates using anti-Flag antibody with Protein A/G Sepharose beads followed by SDS-PAGE and immunoblotting with anti-Myc (upper panel) and anti-Flag (lower panel). Input is 2.5% of IP sample.

(B) Endogenous SREBP-2 interacts with endogenous mutant p53. Nuclear lysates from serum-starved MDA-468.shp53 cells were immunoprecipitated with an anti-SREBP-2 antibody (1D2)

or Mouse IgG (Mock IP) and then immunoblotted with anti-SREBP-2 (1D2) and anti-p53 antibodies (DO-1). Input is 10% of IP sample.

(C) ChIP analysis of mutant p53 in the vicinity of SRE-1 sites. MDA-468.shp53 cells were grown in 2D culture for 8 days in the absence or presence of DOX to deplete mutant p53. Mutant p53 was immunoprecipitated from 1 mg of MDA-468.shp53 lysates using anti-p53 antibodies (1801/DO1). Parallel samples were processed without antibody (Mock IP, "C") to serve as a negative control. Data is presented as mean ± st dev of three independent experiments. Values were normalized to the highest immunoprecipitation signal. **indicates $p<0.01$ or *indicates $p<0.05$ compared to all of the following: negative site, +DOX and Mock IP.

(D) ChIP analysis of mutant p53 at different regions of the *HMGCR* promoter. MDA-468.shp53 cells were grown in 2D culture for 8 days in the absence or presence of DOX to deplete p53. Mutant p53 was immunoprecipitated from 1 mg of MDA-468.shp53 lysates using anti-p53 antibodies (1801/DO1). Parallel samples were processed without antibody (Mock IP, "C") to serve as a negative control. Data is presented as mean ± st dev of three independent experiments. Values were normalized to the highest immunoprecipitation signal. ** indicates $p<0.01$ compared to all of the following: negative site, +DOX, Mock IP, upstream and downstream sites. Genomic locations of PCR primers are illustrated in a schematic of the *HMGCR* promoter. SRE-1 denotes sterol regulatory element.

(E) ChIP analysis of mutant p53 on the *HMGCR* promoter after SREBP2 knockdown. MDA-468 cells were treated with siRNA directed against SREBP2 and subjected to ChIP analysis for

mutant p53 recruitment to the vicinity of the SRE-1 site in the *HMGCR* promoter (-150bp). Data is presented as mean ± st dev of three independent experiments. Values were normalized to the highest immunoprecipitation signal. *designates $p<0.05$. See Figure 2.S6 for extent of SREBP2 knockdown.

(F) ChIP analysis of mutant p53 on the *HMGCR* promoter after SREBP inhibition by Fatostatin. MDA-468.shp53 cells were treated with Fatostatin (20 μM) and subjected to ChIP analysis for mutant p53 recruitment to the vicinity of the SRE-1 site in the *HMGCR* promoter (-150bp). Data is presented as mean ± st dev of six independent experiments. Values were normalized to the highest immunoprecipitation signal. **designates $p<0.01$.

(G) Inhibition of SREBP activation affects MDA-231 cell morphology in 3D cultures. MDA-231.shp53 cells were grown in 3D culture conditions for 8 days treated with vehicle (DMSO), Fatostatin (2 μM) or Fatostatin (20 μM) as indicated. Drugs were added on Day 1. Scale Bar, 200 μm.

Figure 2.7 Mutant p53 is correlated with higher expression of a subset of mevalonate pathway genes in human breast cancer patient datasets

(A) Five human breast cancer patient datasets were analyzed to determine whether tumors bearing mutant p53 correlate with higher expression of sterol biosynthesis genes. Patients were stratified based on *TP53* status (wild-type vs. mutant) and expression levels for sterol biosynthesis genes were analyzed. One of the significantly associated genes, Isopentenyl Pyrophosphate Isomerase (*IDI1*), exhibited higher expression levels in mutant p53 tumors

compared to wild-type p53 tumors ($p<0.05$) across all five datasets. p-value represents the result of a one-sided t-test. See Table 2.S1 for all genes.

(B) Unsupervised hierarchical clustering with Euclidean distance and ward linkage of expression matrix from the 17 sterol biosynthesis genes on 812 samples (728 of which have *TP53* mutational status). *MVD* was not present in the DBCG dataset and its missing expression values were grayed out on the heatmap. Rows indicate the identity of the genes and columns indicate the identity of the patients. The *TP53* mutational status for each tumor is depicted directly above each column. Cluster I exhibits the lowest expression of the mevalonate pathway genes, cluster III exhibits an intermediate expression level and cluster II exhibits the highest expression level of the mevalonate pathway genes.

(C) The Kaplan-Meier curves for the resulting clusters from the unsupervised hierarchical clustering in (B).

(D) Estimated hazard ratios (HRs; the relative risk for 1 unit increasing in the gene expression) with 95% confidence interval for risk of breast cancer specific death. Expression levels of following genes were positively associated with the risk of breast cancer specific death at FDR (q) 5%: *ACAT2* (HR = 1.23, q = 0.0069), *HMGCS1* (HR = 1.21, q = 0.007), *HMGCR* (HR = 1.17, q = 0.032), *IDI1* (HR = 1.26, q < 0.001), *FDPS* (HR = 1.17, q = 0.012), *SQLE* (HR = 1.35, q < 0.001), *LSS* (HR = 1.16, q = 0.032), *NSDHL* (HR = 1.17, q = 0.032), *DHCR7* (HR = 1.26, q < 0.001). Blue indicates $q<0.05$, grey indicates $q>0.05$.

Figure 2.S1 Depletion of mutant p53 from breast cancer cells induces a phenotypic reversion in 3D culture, (Related to Figure 2.1)

(A) Doxycycline curve in MDA-231.shp53 cells. MDA-231.shp53 cells were grown in 2D culture in the presence of the indicated concentrations of DOX for 8 days. p53 was detected using an anti-p53 antibody (PAb1801). Actin serves as a loading control.

(B) Doxycycline curve in MDA-231.shp53 cells in 3D culture. MDA-231.shp53 cells were grown in 3D culture for 8 days in the presence of the indicated concentrations of DOX and imaged using differential interference microscopy. Scale Bar, 200 μm.

(C) Doxycycline curve in MDA-468.shp53 cells. MDA-468.shp53 cells were grown in 2D culture in the presence of the indicated concentrations of DOX for 8 days. p53 was detected using an anti-p53 antibody (PAb1801). Actin serves as a loading control.

(D) Doxycycline curve in MDA-468.shp53 cells in 3D culture. MDA-468.shp53 cells were grown in 3D culture for 8 days in the presence of the indicated concentrations of DOX and imaged using differential interference microscopy. Scale Bar, 200 μm.

(E) Reverted MDA-468.shp53 cells regain proper localization of α6 integrin. MDA-468.shp53 cells well grown in 3D culture for 8 days in the presence (top panels) or absence (bottom panels) of DOX to deplete levels of endogenous mutant p53. Alpha 6 integrin (red) was immunostained using a monoclonal antibody directed against α6 integrin and nuclei were stained with DRAQ5 (Blue). Structures were analyzed by confocal microscopy. Scale bar, 50 μm.

(F) Expression of shRNA-resistant p53-R273H can compensate for depletion of p53-R280K from MDA-231 cells. MDA-231.shp53 cells expressing a control vector were grown in 3D culture for 8 days in the absence of DOX (top left panel), thus retaining full levels of mutant p53, or grown in the presence of DOX (top right panel) to ablate endogenous mutant p53. MDA-231.shp53 cells expressing a shRNA-resistant Flag-tagged version of mutant p53 (p53-R273H) were grown in 3D culture for 8 days in the absence of DOX (bottom left panel), thus retaining full levels of both exogenous and endogenous mutant p53, or grown in the presence of DOX (bottom right panel) to ablate endogenous mutant p53, but retain exogenous p53-R273H. Scale Bar, 200 μm.

(G) Levels of endogenous mutant p53 and retention of exogenous mutant p53. Cells were grown for 8 days in the presence or absence of DOX as indicated. p53 was detected using an anti-p53 antibody (PAb1801). Actin serves as a loading control.

Figure 2.S2 Tumor-derived mutants of p53 disrupt acinar morphogenesis in non-malignant mammary epithelial cells, (Related to Figure 2.2)

(A) Schematic of normal mammary acinar development.

(B-G) Mutant p53 disrupts normal mammary morphogenesis. MCF10A cells expressing an empty vector (B) or Flag-tagged versions of p53-R175H (C), p53-R248W (D), p53-R273H(E), p53-R248Q (F) or p53-G245S (G) were grown in 3D culture for 8 days. Structures were analyzed by confocal microscopy. Nuclei were stained with DRAQ5 (Red). Scale Bar, 50 μm.

(H) Immunoblot for p53 expression demonstrating endogenous wild-type p53 and exogenous Flag-tagged mutants. MCF10A cells expressing Flag-tagged versions of mutant p53 were grown in 3D culture for 8 days. p53 was detected using an anti-p53 antibody (PAb1801). Actin serves as a loading control.

(I) Morphometry of structures. MCF10A cells expressing tumor-derived mutants of p53, or their transactivation-deficient (mTAD) equivalents, were grown in 3D culture for 8 days and analyzed by confocal microscopy for the presence of a hollow or filled lumen. Structures (50-100) were counted for each condition. A representative experiment is shown.

(J) Immunoblot for p53 expression demonstrating exogenous Flag-tagged mutants. MCF10A cells expressing Flag-tagged versions of mutant p53 with wild-type of mutant (mTAD) transactivation domains were grown in 2D culture, lysed and whole cell extracts were subjected to SDS-PAGE and then immunoblotted. Exogenous p53 was detected using an anti-Flag antibody. Actin serves as a loading control.

Figure 2.S3. Schematic of the mevalonate pathway, (Related to Figure 2.3)

(A) Schematic of the mevalonate pathway. Key intermediate metabolites are shown in bold. Gene names are shown in parentheses. Inhibitors are indicated using gray boxes.

Figure 2.S4 Downregulation of the mevalonate pathway is both necessary and sufficient to phenotypically revert breast cancer cells in 3D culture, (Related to Figure 2.4)

(A) Intermediate metabolites rescue the phenotypic effects of depleting breast cancer cells of mutant p53. MDA-468.shp53 cells were grown in 3D culture for 8 days in the presence or absence of DOX to knockdown mutant p53. Cells which were grown in the presence of DOX were then supplemented with two metabolites produced within the mevalonate pathway: mevalonic acid/mevalonic acid-phosphate (MVA/MVAP). Morphological categories were determined for 50-100 structures using confocal microscopy which were then plotted as a percentage of the population. A representative experiment is shown.

(B) Add-back of mevalonic acid/mevalonic acid-phosphate (MVA/MVAP) does not affect mutant p53 depletion by doxycycline. MDA-468.shp53 cells were cultured in the presence of doxycycline to knockdown mutant p53 with or without supplementation of 1mM MVA/MVAP. Whole cell extracts were then subjected to SDS-PAGE and then immunoblotted. p53 was detected using an anti-p53 antibody (PAb1801). Actin serves as a loading control.

(C) Simvastatin treatment does not affect the morphology of MCF10A cells. MCF10A cells were grown in 3D culture for 13 days untreated or treated with vehicle (DMSO), Simvastatin (100 nM) or Simvastatin (1 μM) as indicated. Drugs were added on Day 4. Scale Bar, 200 μm.

(D) Mevastatin profoundly affects the 3D morphology of MDA-231 cells. MDA-231 cells were grown in 3D culture for 13 days. On Day 4, vehicle (DMSO) or Mevastatin (1 μM) were added as indicated for the remainder of the experiment. Scale Bar, 200 μm.

(E) Mevastatin profoundly affects the 3D morphology of MDA-468 cells. MDA-468 cells were grown in 3D culture for 13 days. On Day 4, vehicle (DMSO) or Mevastatin (1 μM) were added as indicated for the remainder of the experiment. Scale Bar, 200 μm.

(F) Inhibition of Mevalonate Decarboxylase affects the 3D morphology of MDA-468 cells. MDA-468 cells were grown in 3D cultures for 8 days. On Day 1, vehicle (DMSO) or 6-Fluoromevalonate (200 μM) was added for the remainder of the experiment. Scale Bar, 200 μm.

(G) Inhibition of Mevalonate Decarboxylase affects the 3D morphology of MDA-231 cells. MDA-231 cells were grown in 3D cultures for 8 days. On Day 1, vehicle (DMSO) or 6-Fluoromevalonate (200 μM) was added for the remainder of the experiment. Scale Bar, 200 μm.

Figure 2.S5 Simvastatin prevents growth in breast cancer cells *in vivo* and induces a cell cycle arrest in cells grown in 2D culture, (Related to Figure 2.5)

(A) Simvastatin prevents anchorage-independent growth in breast cancer cells. MDA-231 or MDA-468 cells were grown in soft-agar for 21 days in the presence of DMSO vehicle control or presence of Simvastatin (0.1, 1, 10 μM). Plates were subsequently stained with crystal violet and colonies were counted for each condition. Quantitation of three independent experiments illustrating relative colony number for MDA-231 (left) and MDA-468 (right) cells. Data presented as mean ± st dev. *denotes $p<0.05$, **denotes $p<0.01$ using a two-tailed students t-test.

(B-C) Simvastatin induces a G1 arrest in breast cancer cells grown in 2D culture. Flow cytometric analysis of cell cycle distribution for three independent experiments in MDA-468

cells (B) or MDA-231 cells (C). Data presented as mean ± st dev. *denotes $p<0.05$, **denotes $p<0.01$ using a two-tailed students t-test.

(D) Simvastatin significantly impacts tumor growth *in vivo*. MDA-231 cells ($2x10^6$) were injected subcutaneously into 8 week-old NOD-SCID mice. Fourteen days after implantation mice were paired by equal tumor volumes and randomized to either a Simvastatin (200 mg/kg/day) or Control (placebo) group (N=5 for each group). Tumor measurements were performed weekly using calipers. After 21 days of treatment, mice were sacrificed and tumors were extracted and weighed. Tumor volumes as a function of time (left) and tumor weights at day 21 (right) are presented. *denotes $p<0.01$, **denotes $p<0.001$ using a two-tailed students t-test.

Figure 2.S6 Mutant p53 regulates SREBP target genes in breast cancer cells, (Related to Figure 2.6)

(A) Endogenous SREBP-2 interacts with endogenous mutant p53. Nuclear lysates from serum-starved MDA-231.shp53 cells were immunoprecipitated with an anti-SREBP-2 antibody (1D2) or Mouse IgG and then immunoblotted with anti-SREBP-2 (1D2) and anti-p53 antibodies (DO-1). Input is 10% of IP sample.

(B) Venn diagram illustrating overlap between SREBP target genes and genes changed after mutant p53 knockdown. Significant gene expression changes ($p<0.05$) from genome-wide expression analysis of MDA-468 cells depleted of mutant p53 were queried against a

comprehensive list of SREBP1 target genes (Reed et al., 2008). P-value was determined by the Chi-squared method.

(C) Sterol biosynthesis genes regulated by mutant p53. MDA-468.shp53 cells were grown in 3D culture for 8 days in the presence or absence of DOX to knockdown mutant p53. qRT-PCR of three independent experiments of sterol biosynthesis genes not initially identified using IPA. Data presented as mean ± st dev. *indicates $p<0.05$, ** indicates $p<0.01$ using a two-tailed t-test.

(D) Sterol biosynthesis genes regulated by mutant p53 in MDA-231 cells. MDA-231.shp53 cells were grown in 3D culture for 8 days in the presence or absence of DOX to knockdown mutant p53. qRT-PCR of three independent experiments of sterol biosynthesis genes. Data presented as mean ± st dev. *indicates $p<0.05$ using a two-tailed t-test.

(E) SREBP target genes, including fatty acid biosynthesis genes, regulated by mutant p53. MDA-468.shp53 cells were grown in 3D culture for 8 days in the presence or absence of DOX to knockdown mutant p53. qRT-PCR of three independent experiments of SREBP target genes. Data presented as mean ± stdev. **indicates $p<0.01$ using a two-tailed t-test.

(F) Immunoblot demonstrating knockdown of SREBP2. MDA-468 cells were treated with a control siRNA or siRNA directed against SREBP1, SREBP2 or p53 as in Figure 2.6. Whole cell lysates were then subjected to SDS-PAGE. Full-length SREBP2 was detected using an anti-SREBP2 antibody (1D2) and p53 was detected using an anti-p53 antibody (PAb1801). Actin serves as a loading control.

(G) ChIP analysis of mutant p53 on the *HMGCR* promoter after SREBP1 knockdown. MDA-468 cells were treated with siRNA directed against SREBP1 and subjected to ChIP analysis for mutant p53 recruitment to the vicinity of the SRE-1 site in the *HMGCR* promoter (-150bp). Data is presented as mean ± st dev of three independent experiments. Values were normalized to the highest immunoprecipitation signal. *designates $p<0.05$.

(H) ChIP analysis of mutant p53 on the *HMGCR* promoter after p53 knockdown. MDA-468 cells were treated with siRNA directed against p53 and subjected to ChIP analysis for mutant p53 recruitment to the vicinity of the SRE-1 site in the *HMGCR* promoter (-150bp). Data is presented as mean ± st dev of three independent experiments. Values were normalized to the highest immunoprecipitation signal. **designates $p<0.01$. See Figure 2.S5C for extent of p53 knockdown.

Figure 2.S7 Mutant p53 is correlated with higher expression of a subset of mevalonate pathway genes in human breast cancer patient datasets, (Related to Figure 2.7)

Five human breast cancer patient datasets were analyzed to determine whether tumors bearing mutant p53 correlate with higher expression of sterol biosynthesis genes. Patients were stratified based on *TP53* status (wild-type vs. mutant) and expression levels for sterol biosynthesis genes were analyzed. p-value represents the result of a one-sided t-test. See Table 2.S1 for all genes.

(A) Farnesyl Diphosphate Synthase (*FDPS*) exhibited higher expression levels in mutant p53 tumors compared to wild-type p53 tumors ($p<0.05$) in three out of five datasets.

(B) Squalene Epoxidase (*SQLE*) exhibited higher expression levels in mutant p53 tumors compared to wild-type p53 tumors ($p<0.05$) in four out of five datasets.

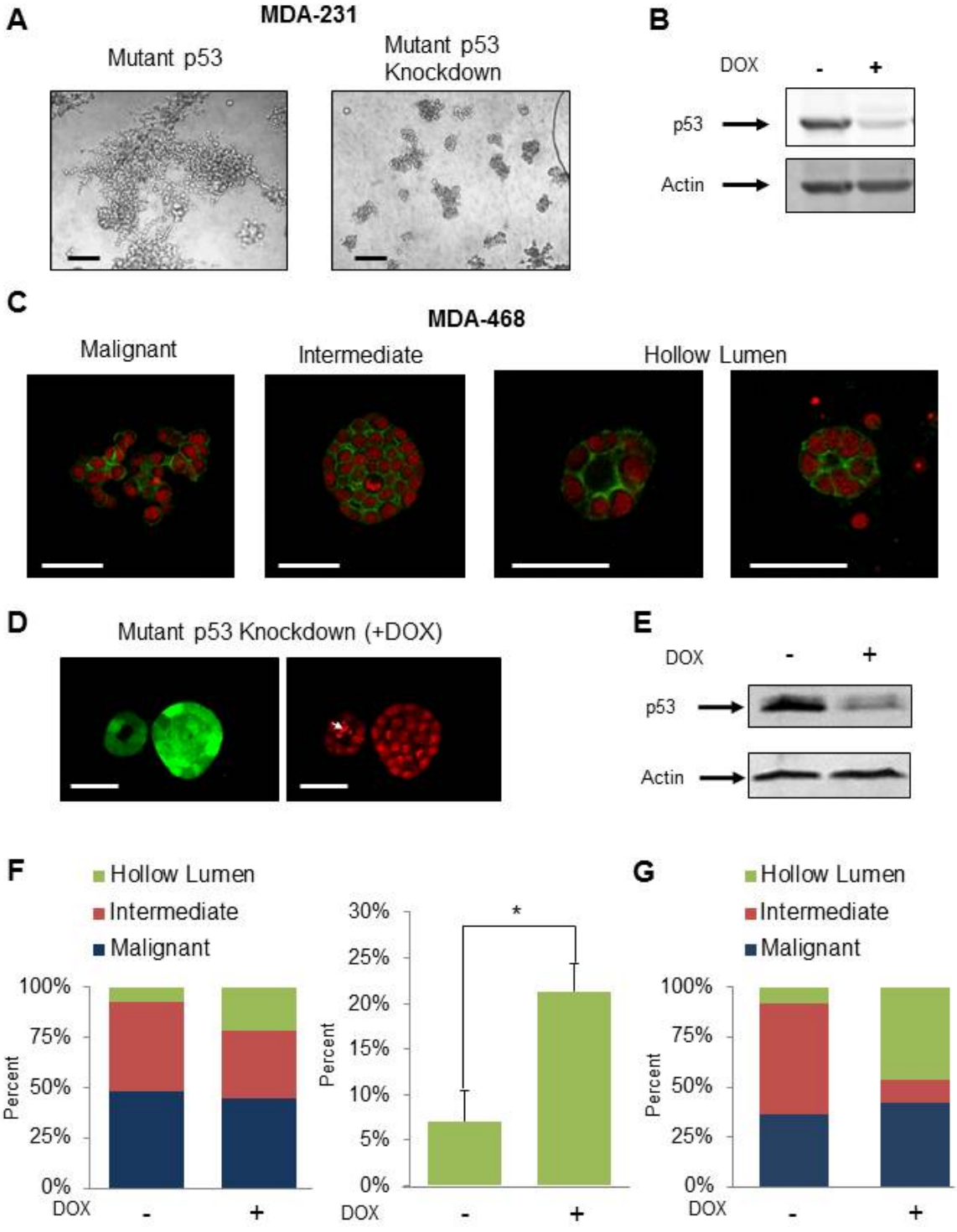
(C) 7-Dehydrocholesterol reductase (*DHCR7*) exhibited higher expression levels in mutant p53 tumors compared to wild-type p53 tumors ($p<0.05$) across all five datasets.

Table 2.S1 Mutant p53 is correlated with higher expression of a subset of mevalonate pathway genes in human breast cancer patient datasets, (Related to Figure 2.7)

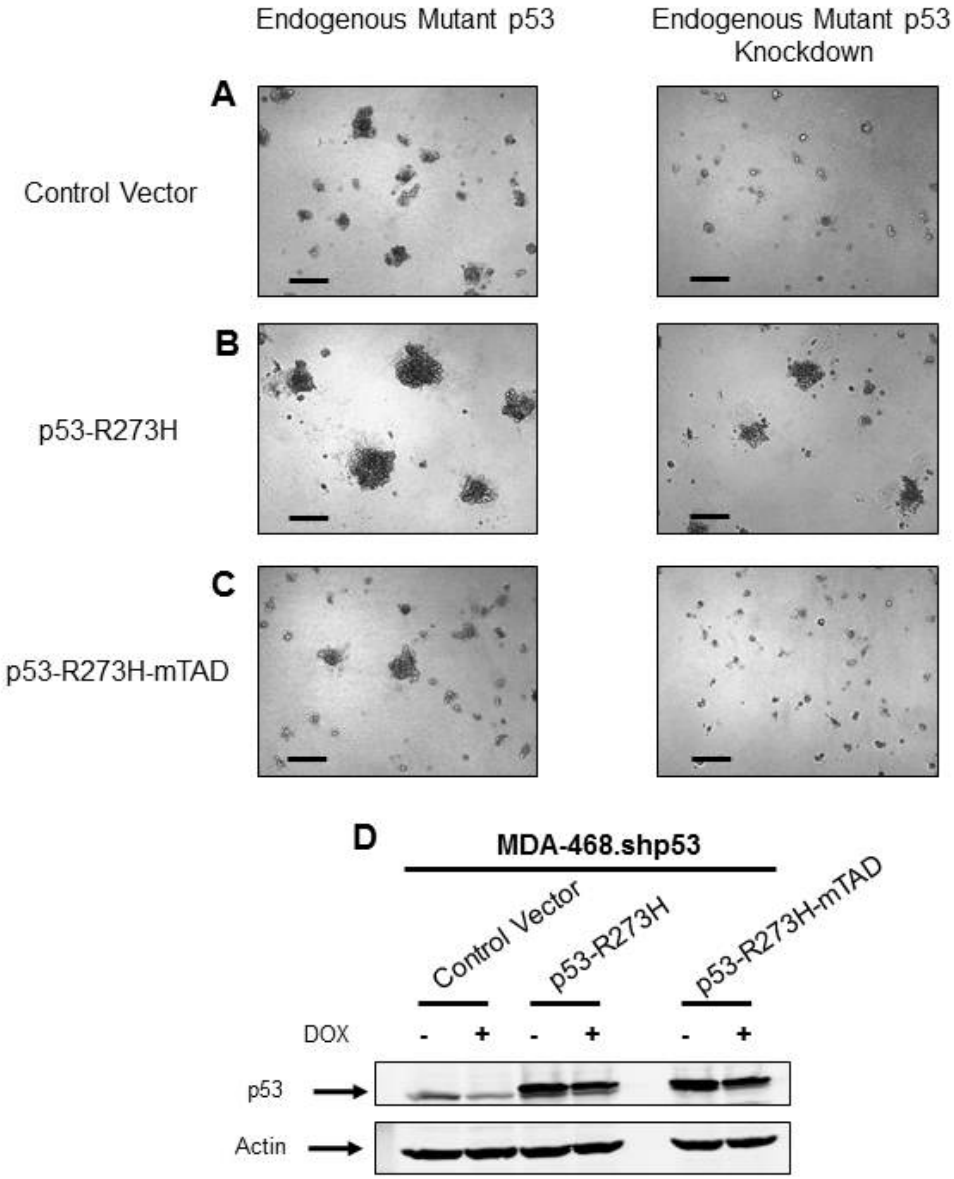
(A) Five human breast cancer patient datasets, FW-MDG, MicMa, ULL, DBCG and Miller were analyzed to determine whether tumors bearing mutant p53 correlated with higher expression of sterol biosynthesis genes. Patients were stratified based on *TP53* status (wild-type vs. mutant) and expression levels for sterol biosynthesis genes were analyzed. p-value represents the result of a one-sided t-test for seventeen sterol biosynthesis genes. The right-hand column provides the False Discovery Rate (FDR) for each gene across the five datasets.

Table 2.S2 Primer Sequences

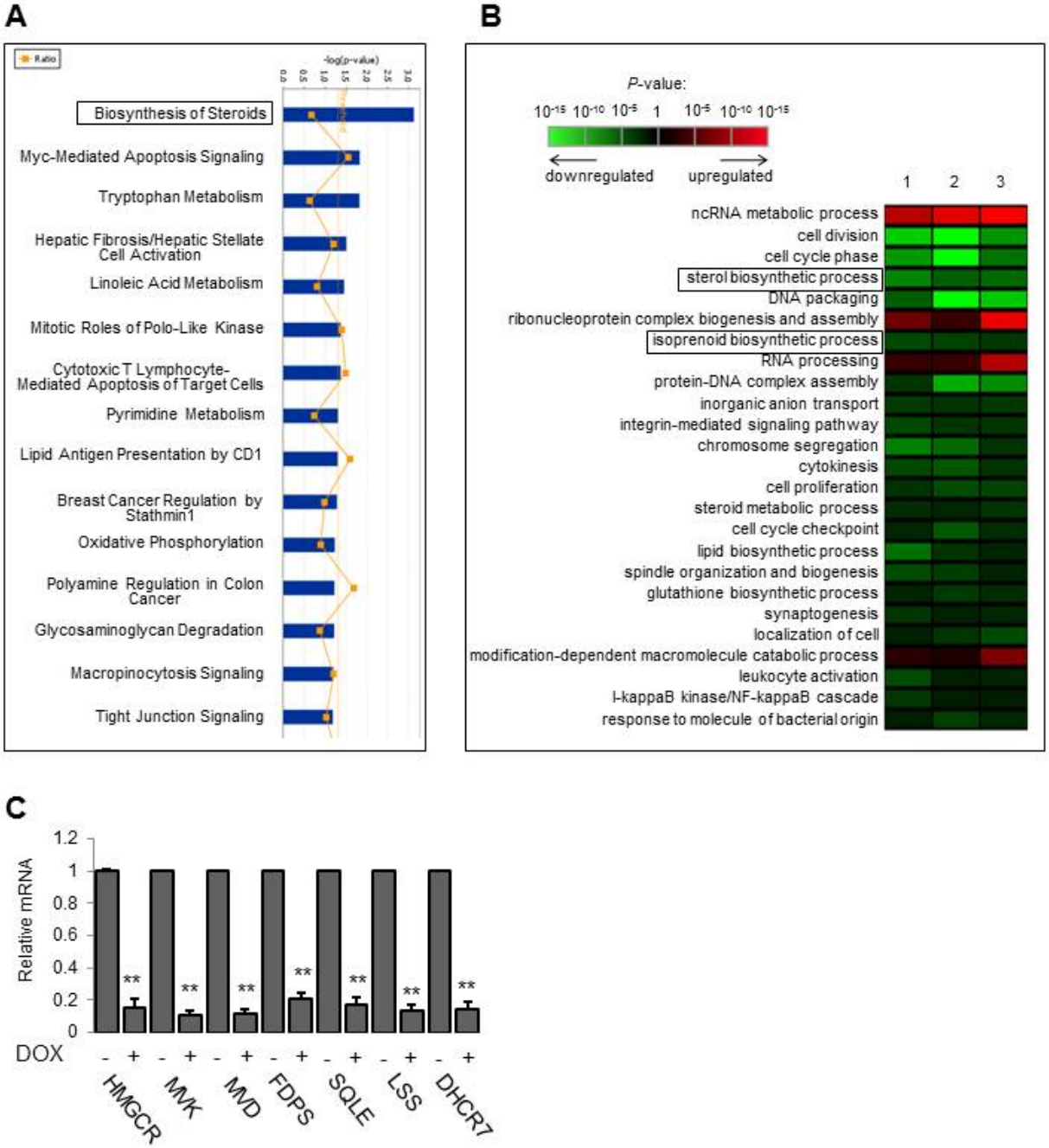
Freed-Pastor et al., Figure 2.1



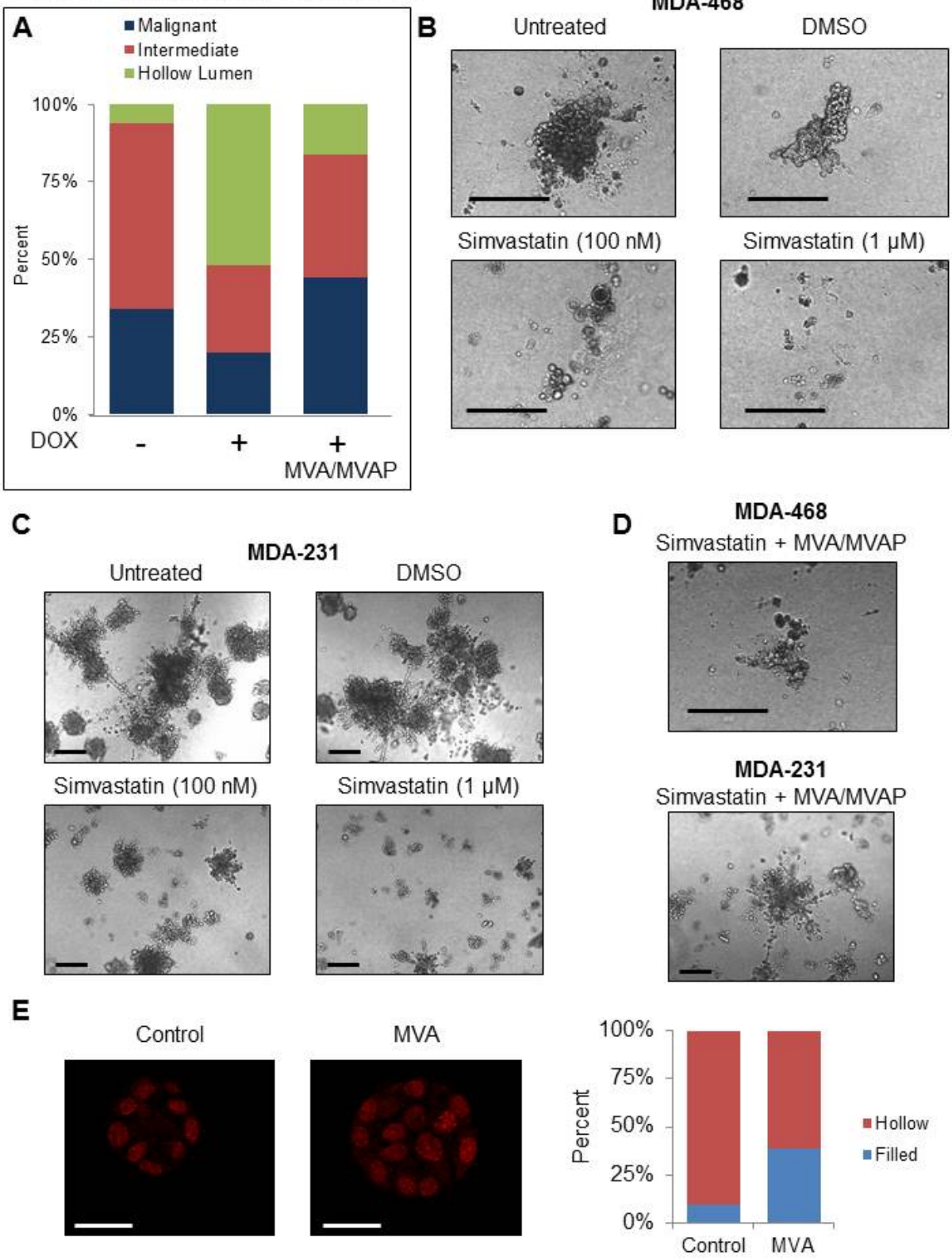
Freed-Pastor et al., Figure 2.2



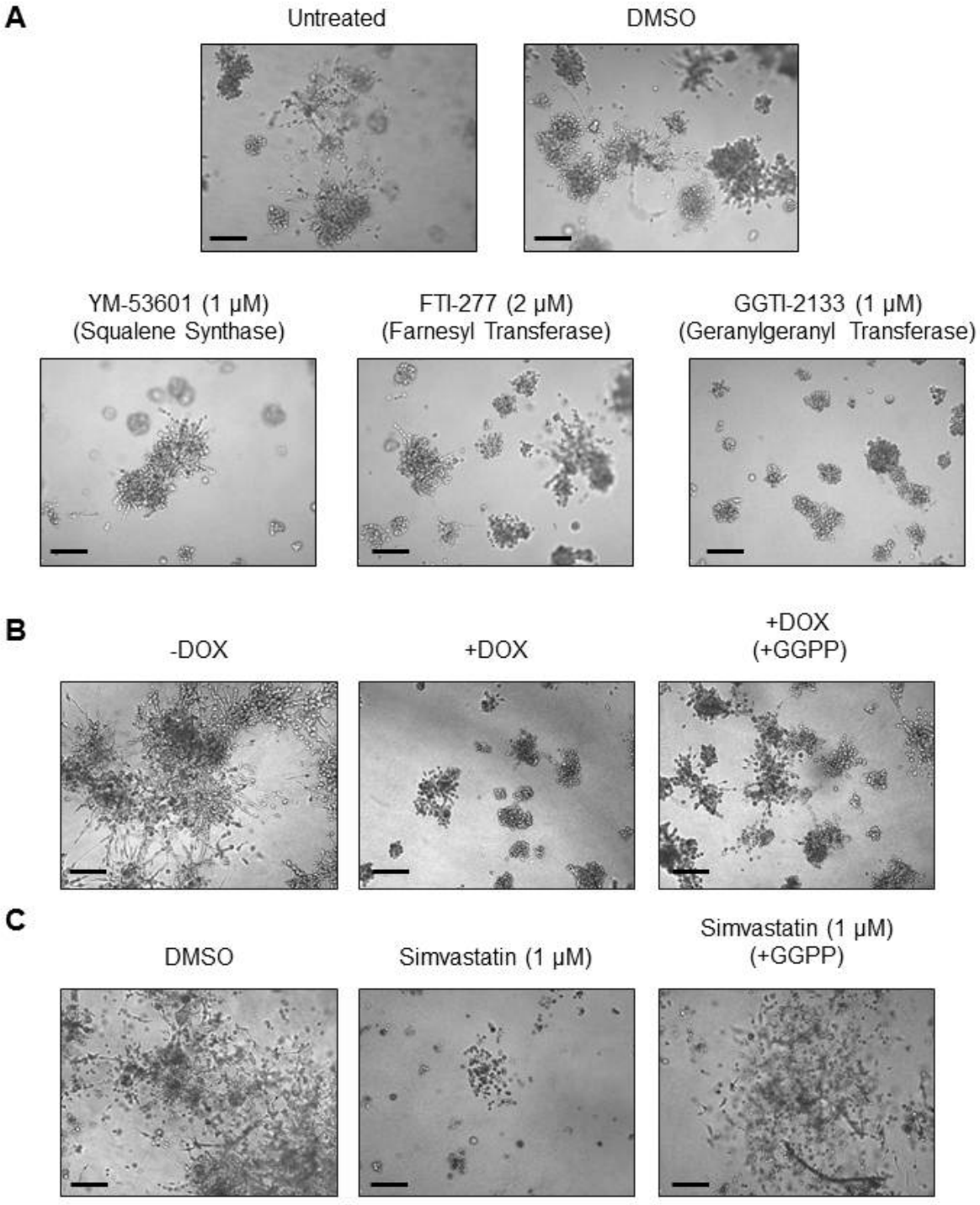
Freed-Pastor et al., Figure 2.3



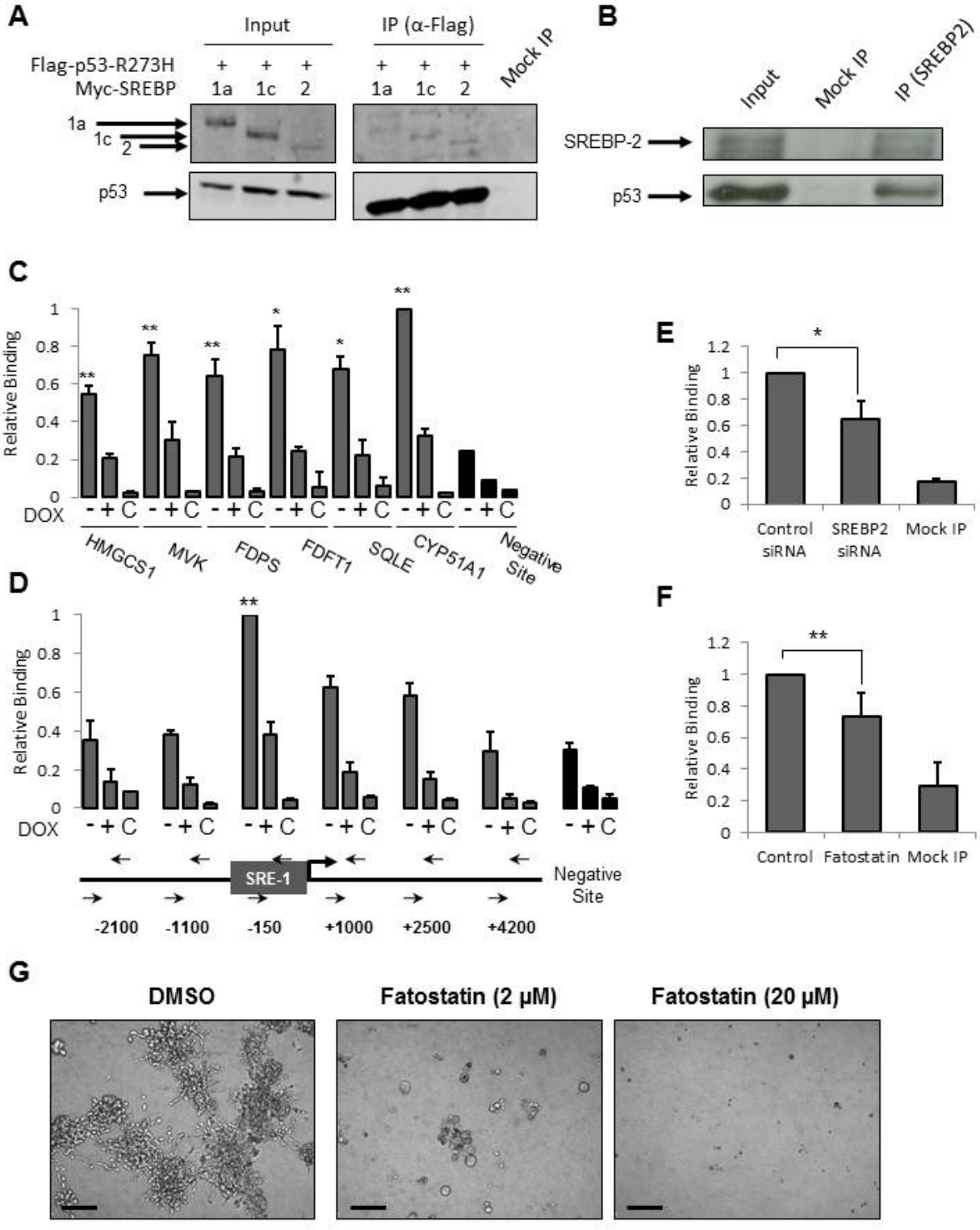
Freed-Pastor et al., Figure 2.4



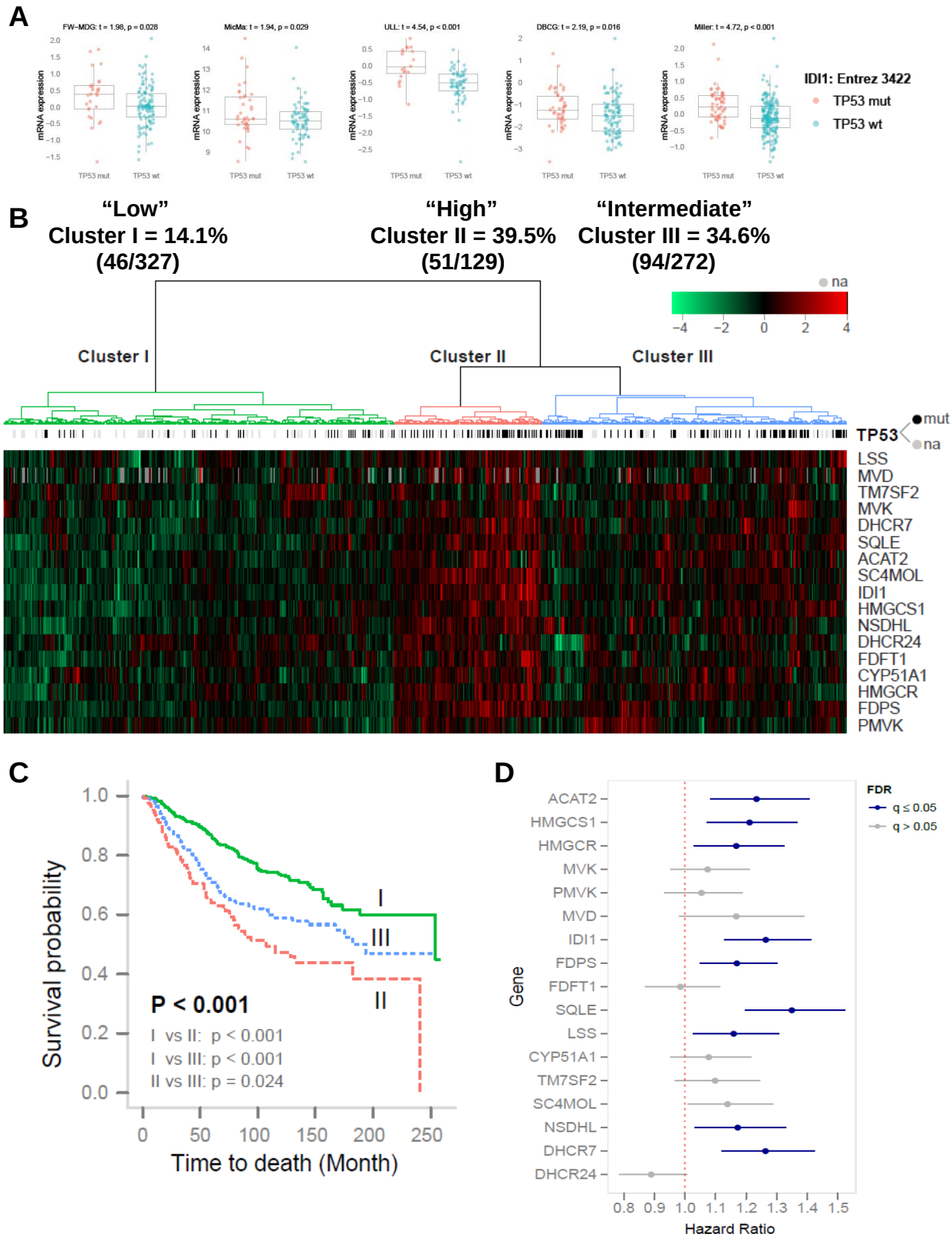
Freed-Pastor et al., Figure 2.5

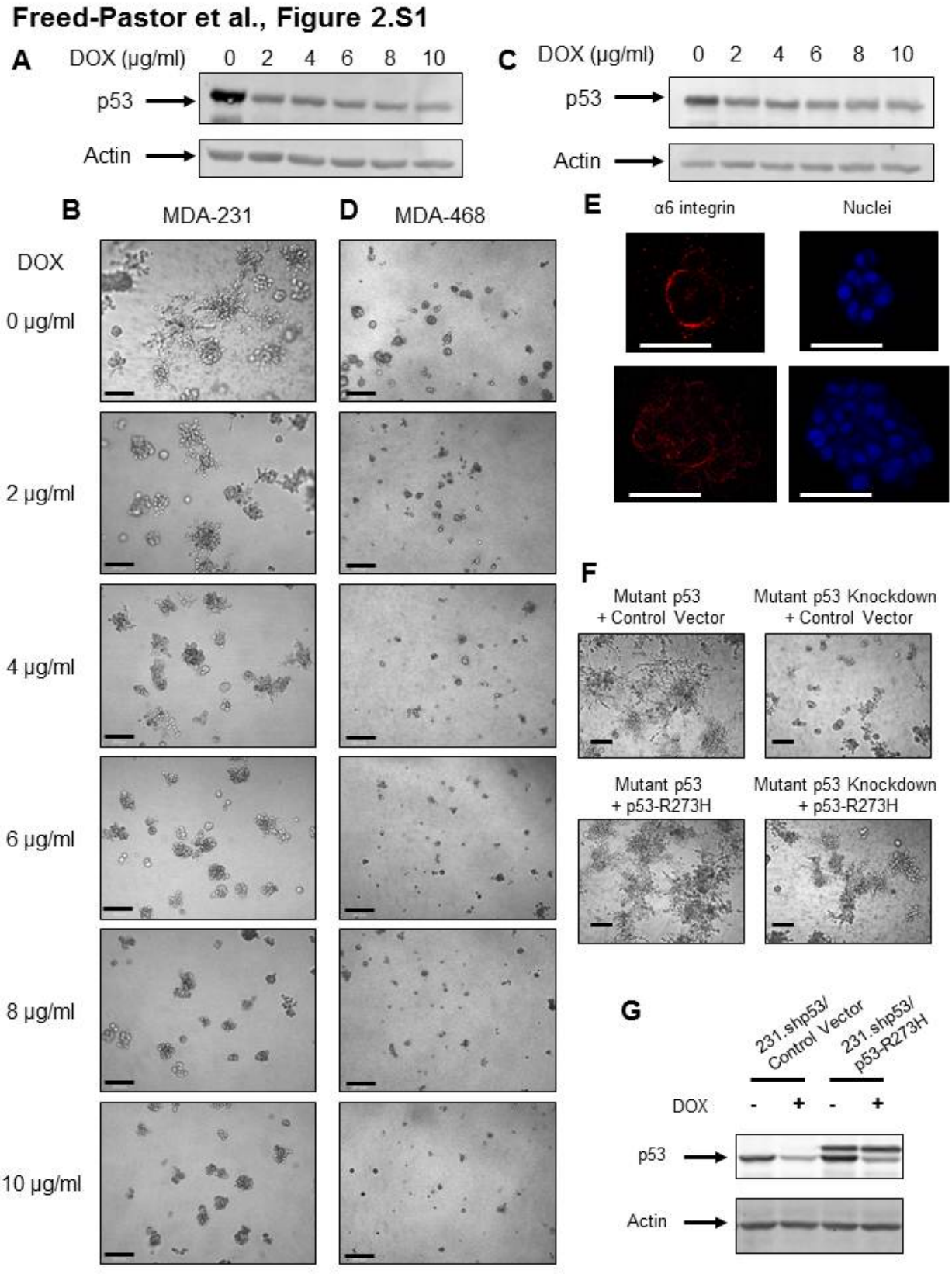


Freed-Pastor et al., Figure 2.6

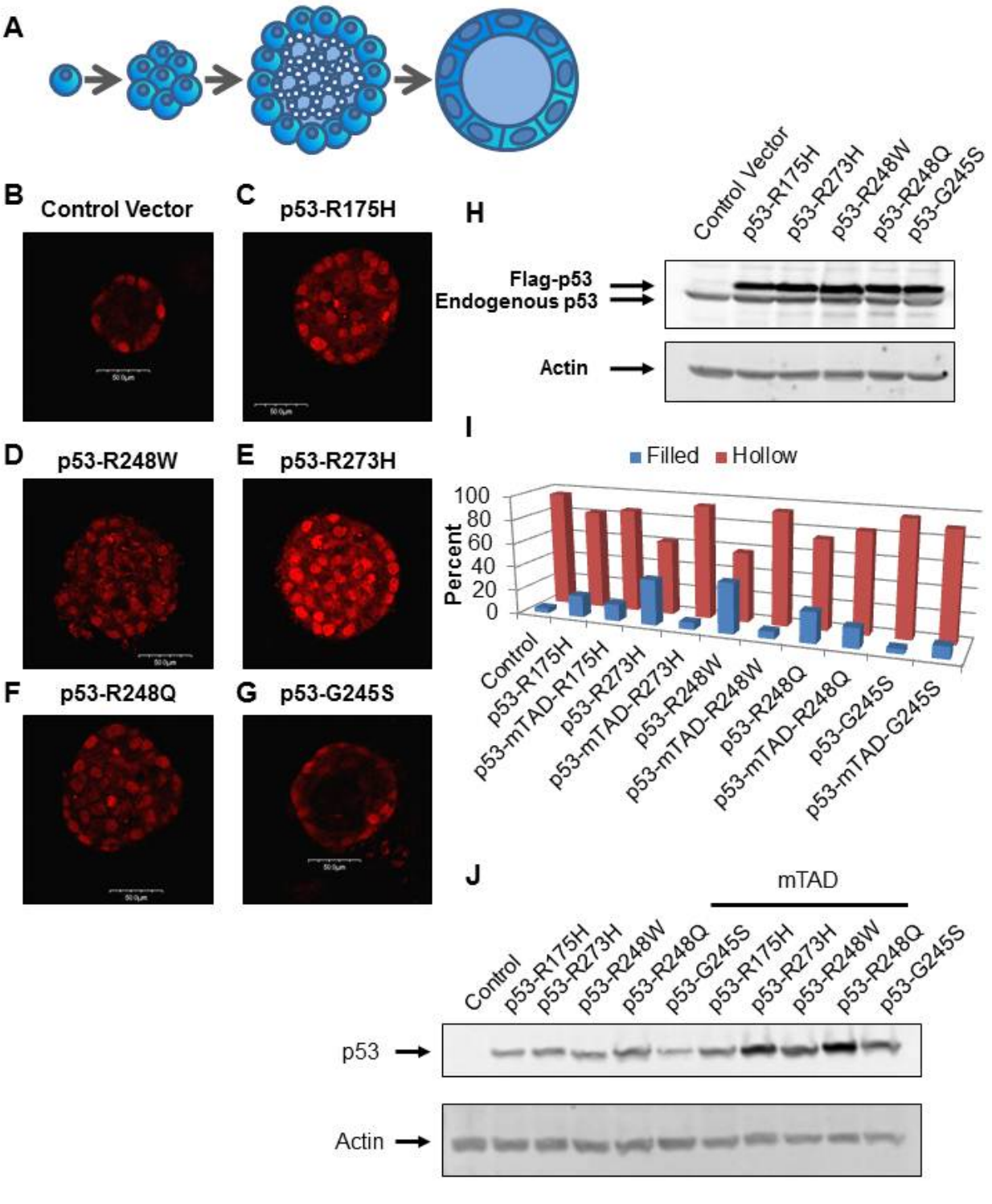


Freed-Pastor et al., Figure 2.7



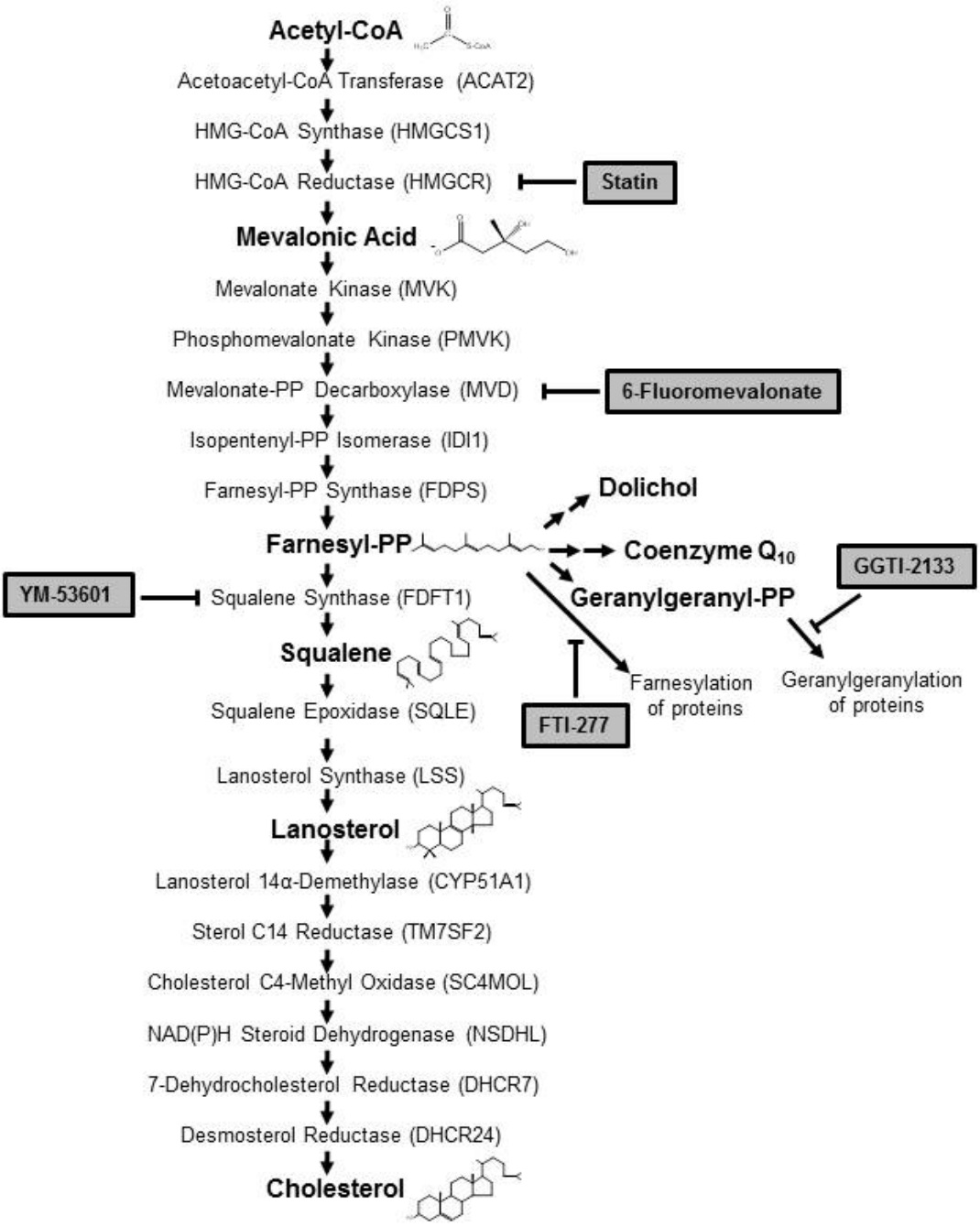

Freed-Pastor et al., Figure 2.S1
A
DOX (µg/ml) 0 2 4 6 8 10
p53
Actin
C
DOX (µg/ml) 0 2 4 6 8 10
p53
Actin
B
MDA-231
D
MDA-468
DOX
0 µg/ml
2 µg/ml
4 µg/ml
6 µg/ml
8 µg/ml
10 µg/ml
E
α6 integrin
Nuclei
F
Mutant p53 + Control Vector
Mutant p53 Knockdown + Control Vector
Mutant p53 + p53-R273H
Mutant p53 Knockdown + p53-R273H
G
231.shp53/ Control Vector
231.shp53/ p53-R273H
DOX - + - +
p53
Actin

Freed-Pastor et al., Figure 2.S2



Freed-Pastor et al., Figure 2.S3

A



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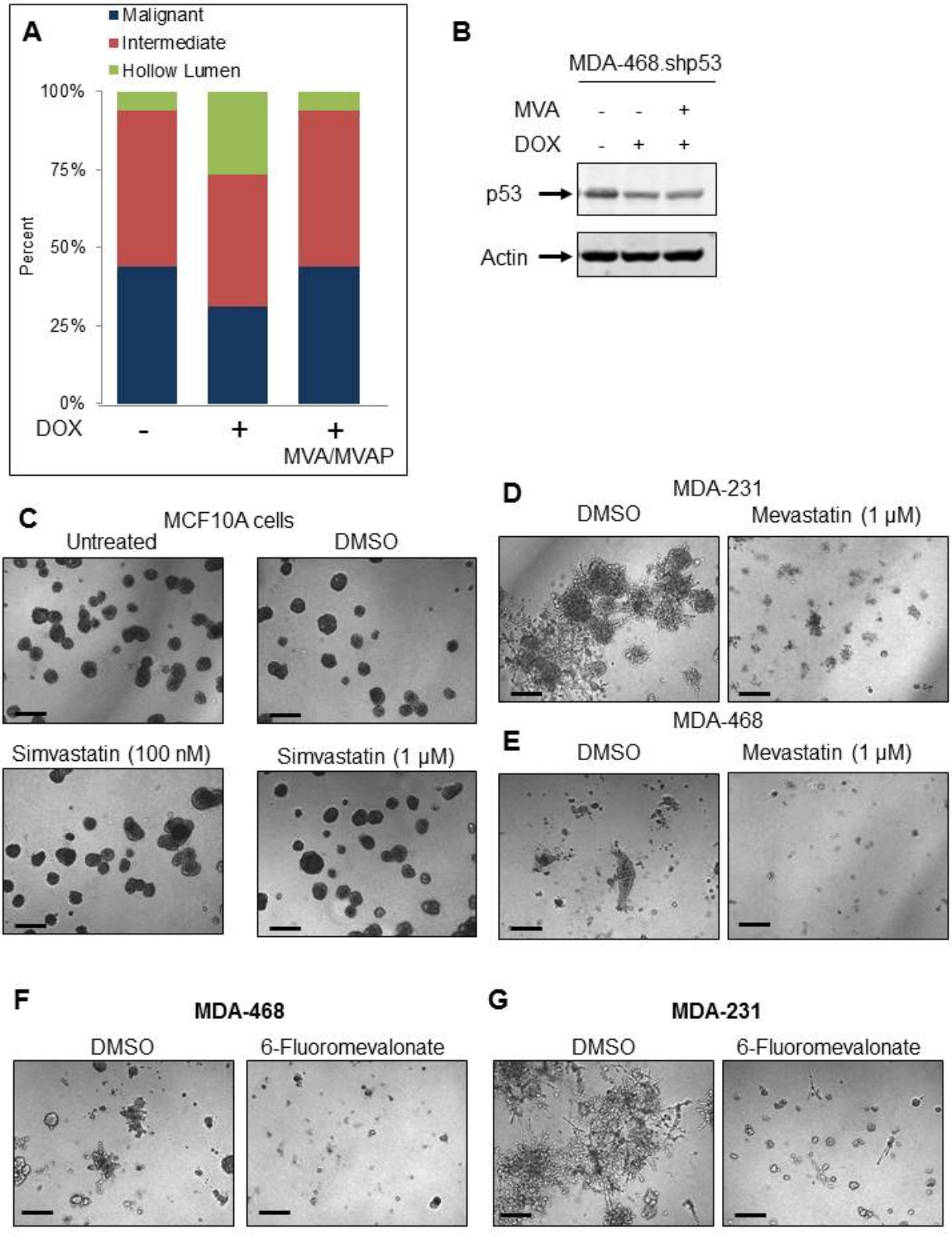
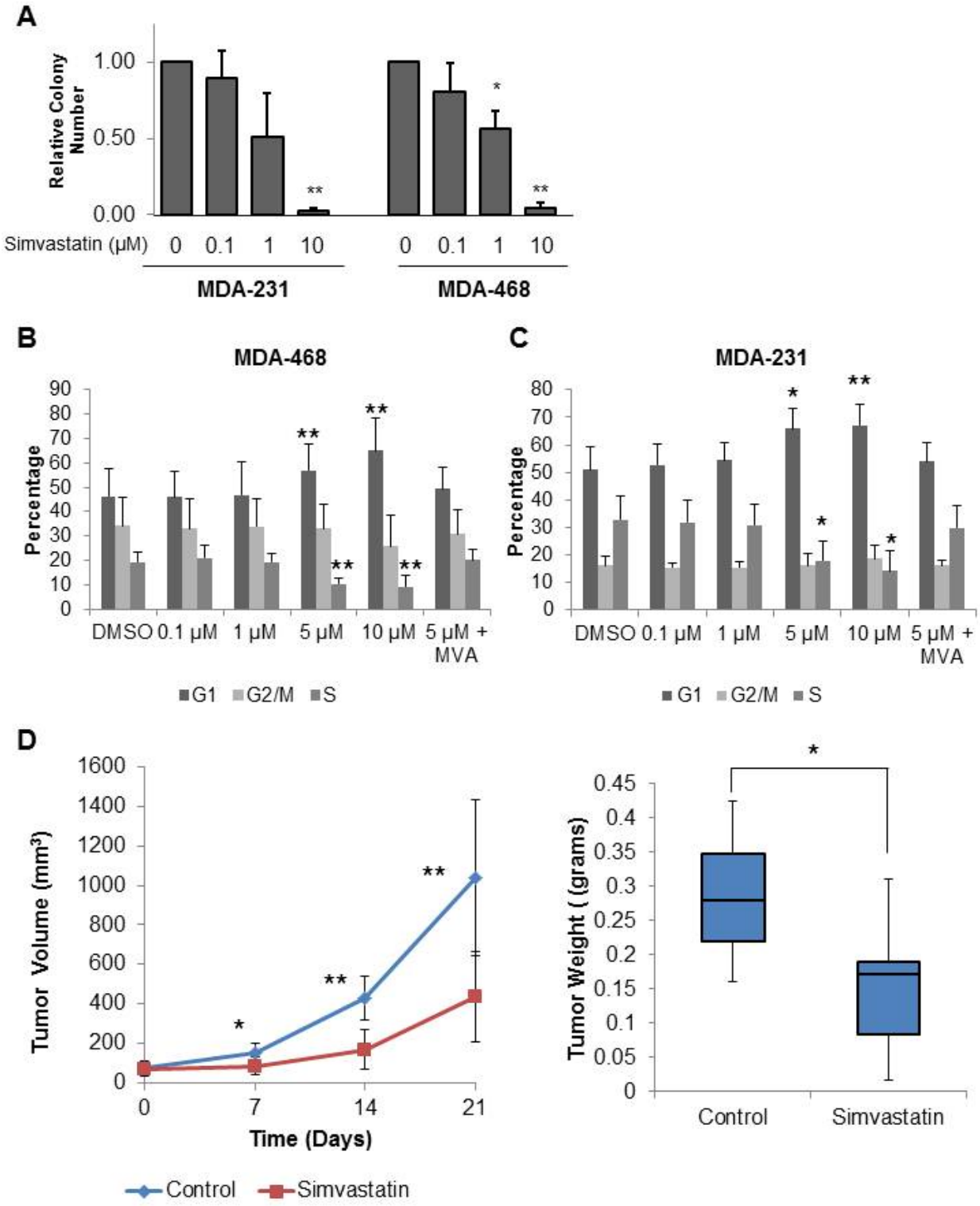
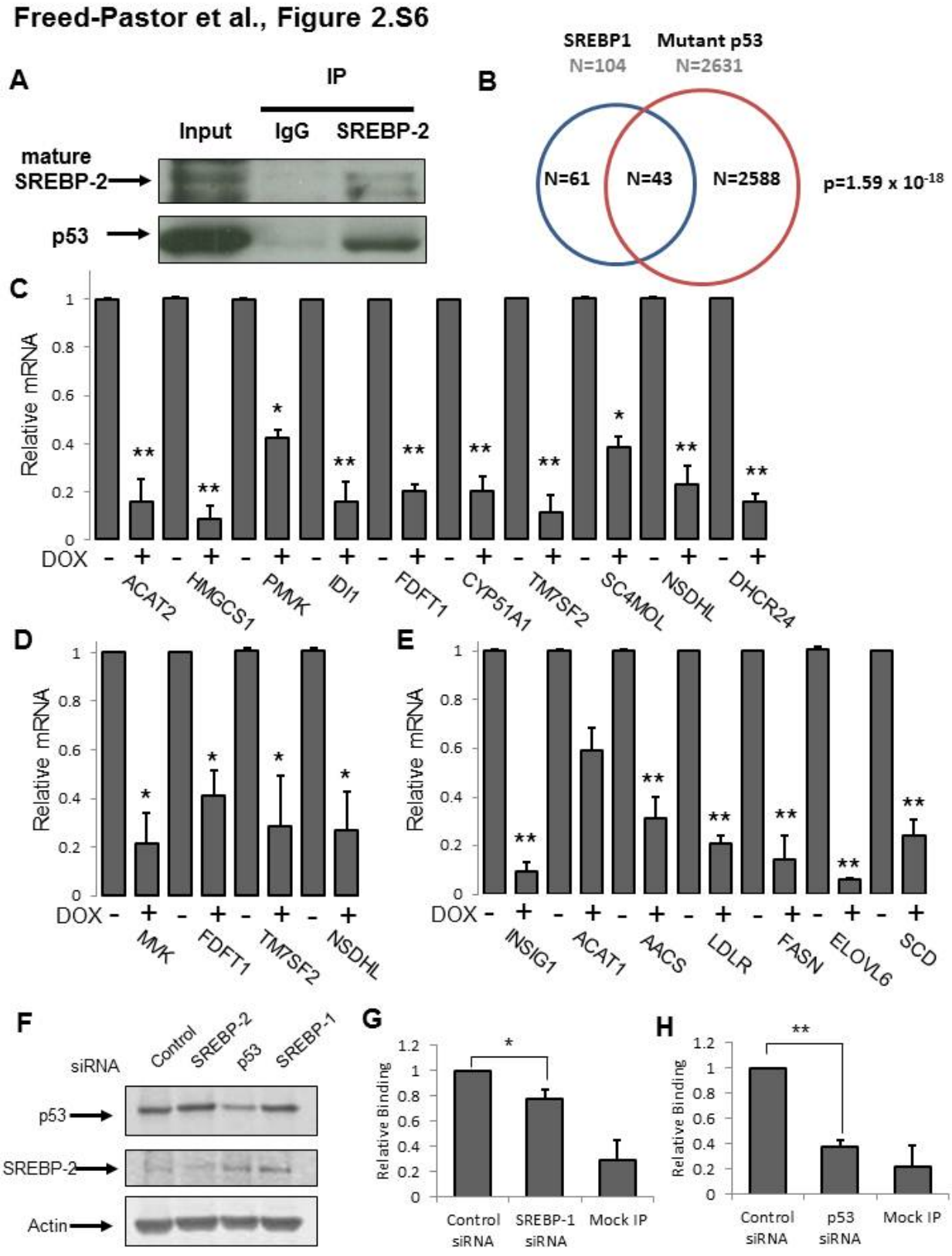
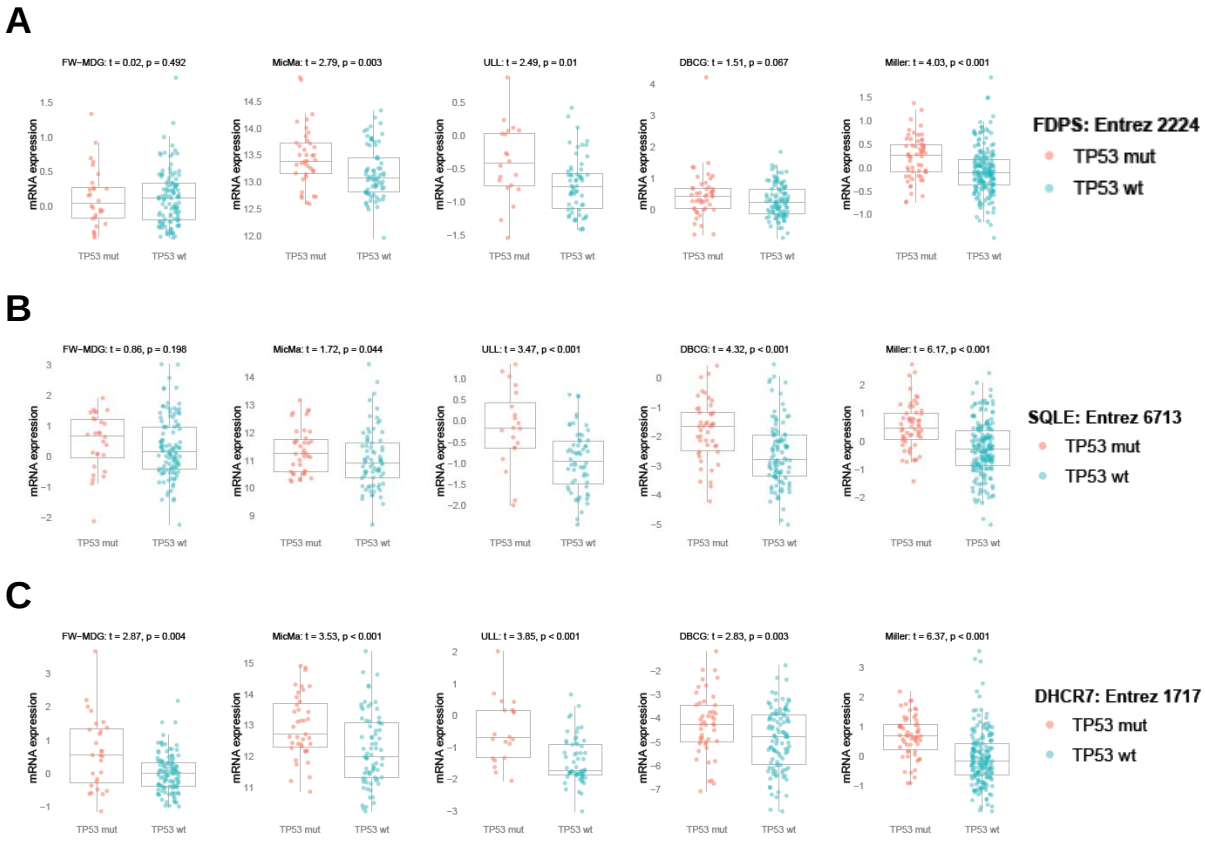


Freed-Pastor et al., Figure 2.S5




Freed-Pastor et al., Figure 2.S6
A
IP
Input
IgG
SREBP-2
mature SREBP-2
p53
B
SREBP1 N=104
Mutant p53 N=2631
N=61
N=43
N=2588
p=1.59 x 10-18
C
Relative mRNA
DOX
ACAT2
HMGCS1
PMVK
IDI1
FDFT1
CYP51A1
TM7SF2
SC4MOL
NSDHL
DHCR24
D
Relative mRNA
DOX
MVK
FDFT1
TM7SF2
NSDHL
E
Relative mRNA
DOX
INSIG1
ACAT1
AACS
LDLR
FASN
ELOVL6
SCD
F
siRNA
Control
SREBP-2
p53
SREBP-1
p53
SREBP-2
Actin
G
Relative Binding
Control siRNA
SREBP-1 siRNA
Mock IP
H
Relative Binding
Control siRNA
p53 siRNA
Mock IP

Freed-Pastor et al., Figure 2.S7



Freed-Pastor et al., Table 2.S1

Gene Symbol	FW-MDG	MicMa	ULL	DBCG	Miller	fisher_p	False Detection Rate
ACAT2	0.011748	0.347815	0.704917	0.003674	2.25E-08	8.16E-09	**4.08E-08**
HMGCS1	0.127913	0.15145	0.007134	0.001947	2.02E-05	1.20E-07	**4.80E-07**
HMGCR	0.11815	0.752179	0.837145	0.367862	0.092586	0.288012	0.384015
MVK	0.157677	0.100831	0.009703	0.022681	0.453415	0.002896	**0.005265**
PMVK	0.955099	0.994949	0.696255	0.990954	0.998388	0.99992	0.99992
MVD	0.00392	0.000109	0.065227	NA	0.004984	3.10E-07	**1.03E-06**
IDI1	0.027657	0.028669	3.81E-05	0.015609	3.88E-06	1.14E-10	**7.62E-10**
FDPS	0.492438	0.003366	0.009796	0.067343	5.30E-05	8.89E-07	**2.54E-06**
FDFT1	0.085465	0.566364	0.365131	0.541292	0.751081	0.451846	0.564807
SQLE	0.198428	0.043982	0.000874	1.97E-05	5.97E-09	1.23E-13	**1.23E-12**
LSS	0.291891	0.034423	0.053203	0.304716	0.000391	0.000258	**0.000573**
CYP51A1	0.473917	0.444983	0.104892	0.636533	0.009544	0.057907	0.089087
TM7SF2	0.653223	0.896114	0.8571	0.553747	0.440326	0.937773	0.99992
SC4MOL	0.621928	0.095479	0.438532	0.200135	0.001093	0.007209	**0.012015**
NSDHL	0.107219	0.12337	0.013431	0.007662	0.001344	1.54E-05	**3.85E-05**
DHCR7	0.003674	0.000334	0.000355	0.002898	1.88E-09	5.55E-16	**1.11E-14**
DHCR24	0.709416	0.625622	0.803548	0.985516	0.993919	0.995484	0.99992

Freed-Pastor et al., Table 2.S2

qRT-PCR			
Gene Symbol		Forward Primer	Reverse Primer
RPL32		TTCCTGGTCCACAACGTCAAG	TGTGAGCGATCTCGGCAC
ACAT2		GCCCCCGCCGACCAT	CCTCCACACGAACACCACCTA
HMGCS1		GGGCAGGGCATTATTAGGCTAT	TTAGGTTGTCAGCCTCTATGTTGAA
HMGCR		GGCCCAGTTGTGCGTCTT	CGAGCCAGGCTTTCACTTCT
MVK		TGGACCTCAGCTTACCCAACA	GACTGAAGCCTGGCCACATC
PMVK		CCGCGTGTCTCACCCTTT	GACCGTGCCCTCAGCTCAT
MVD		TGAACTCCGCGTGCTCATC	CGGTACTGCCTGTCAGCTTCT
IDI1		TTTCCAGGTTGTTTTACGAATACG	TCCTCAAGCTCGGCTGGAT
FDPS		CTTCCTATAGCTGCAGCCATGTAC	GCATTGGCGTGCTCCTTCT
FDFT1		TCAGACCAGTCGCAGTTTCG	CTGCGTTGCGCATTTCC
SQLE		CGTGCTCCTCTTGGTACCTCAT	CGGTCAAGGCGGAGATTATC
LSS		TGCAGAAGGCTCATGAGTTCCT	TCTGGTAGTCGGGAGGGTTATC
CYP51A1		TGCAGCCTGGCTCTTACCA	AGCTCTGTCCCTGCGTCTGA
TM7SF2		GCCACCCTCACCGCTTT	GCTACCTGCGCCTTCATGTAG
SC4MOL		GAAAAGCCGGCACCAAGA	TCAAAGAGAGAATCAGCTCAAACTG
NSDHL		AGAATCAGGCCAAGAGATGCA	TGTGCTGCCCCAGGAATC
DHCR7		GGCATCCCAGCTCCAACTC	GGGCTCTCTCCAGTTTACAGATGA
DHCR24		CAAGTACGGCCTGTTCCAACA	CGCACAAAGCTGCCATCA
INSIG1		CCCAACACCTGGCATCATC	ACCACCCCAACCGAGAAGAG
ACAT1		GCAGCGAAGAGGCTCAATG	GCAGCGTCAGCAAATGCTACT
AACS		ACCCACTGTTCATCATGTTCTCAT	CGGAATGCACCATGCACTT
FASN		CGCTCGGCATGGCTATCT	CTCGTTGAAGAACGCATCCA
LDLR		AAGCCATTCACTTCCCCAATC	GCCTCACCGTGCATGTTTTA
ELOVL6		CCAGTCAACTCCTCGCACTTT	TGACCGTGTCCGGTATTTCC
SCD		CGGGCGGCAGGTTTC	CTGGGACAAGGTGATGAACATG
ChIP			
Gene Symbol	(Relative to TSS)	Forward Primer	Reverse Primer
HMGCR	(-2100)	GAGGAAGCGGCACATGGA	TGGTATGGACACAAGGTAGAAAGG
HMGCR	(-1100)	TTTTCAAGGTCGGGAGTGATG	ACTTTTTCATATGCCACCTCCTTT
HMGCR	(-150)	TGGGACTCGAACGGCTATTG	GAACAGGCACCGCACCAT
HMGCR	(+1000)	GCAGAGTCGTAGGAAGCATTTGT	TGGGACGCCGAAATCATG
HMGCR	(+2500)	GATGAAGGTGGACGATTGAATTC	CCGTTGCCCTGTGATTACG
HMGCR	(+4200)	TTGGTCTTTCCCCTAACCCTTT	AACTGCCACTCTAGCAAGAATTCA
HMGCS1	(-115)	GAGCCAAAATCCTACCGACTCA	ACTCCCGCCTTCTACCAATCAAA
MVK	(-80)	CACTCCCAGGGACTTGTTTCC	GCCGACACGGGTTTTCC
FDPS	(-260)	CAGCTGCCCAGGAAGATAATG	CCCCGCTGTGGCTTTG
FDFT1	(-140)	CTCCAATGAGCTTCTAGAGTGTTATCA	GGAAGACCCCGGCCAAT
SQLE	(-450)	GCGGAATGAATGGAAACGTT	TTGAGGAGAAGCCTGGAGTGA
CYP51A1	(-190)	GCACCCGGGCACACAA	AGGCGATCAATCCCTGAGAA
CDKN1A ("Negative Site")	(+11443)	TCTGTCTCGGCAGCTGACAT	ACCACAAAAGATCAAGGTGAGTGA
Site-Directed Mutagenesis			
Gene Symbol	Mutant	Forward Primer	Reverse Primer
TP53	R175H	ACGGAGGTTGTGAGGCACTGCCCCCACCATGAGCGC	GCGCTCATGGTGGGGGCAGTGCCTCACAACCTCCGT
TP53	R273H	AACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGG	CCCAGGACAGGCACAAACATGCACCTCAAAGCTGTT
TP53	R248W	ATGGGCGGCATGAACTGGAGGCCCATCCTCACC	GGTGAGGATGGGCCTCCAGTTCATGCCGCCCAT
TP53	R248Q	ATGGGCGGCATGAACCAGAGGCCCATCCTCACC	GGTGAGGATGGGCCTCTGGTTCATGCCGCCCAT
TP53	C245S	ACTTCCTGCATCCGCTCCATCAACCCGACCCCC	CCCCCTCCGCTTCATCCACCCCATGCACCAACT
TP53	L22Q, W23S	CTCTGAGTCAGGAAACATTTTCAGACCAATCGAAACTACTTCCTG	CAGGAAGTAGTTTCGATTGGTCTGAAAATGTTTCCTGACTCAGAG
TP53	W53Q, F54S	CCCCGGACGATATTGAACAACAGTCCACTGAAGACCCAGGTCC	GGACCTGGGTCTTCAGTGGACTGTTGTTCAATATCGTCCGGGG

Chapter 3

Mutant p53 regulates integrin β4 to disrupt mammary tissue architecture

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ABSTRACT

The p53 protein plays a pivotal role in myriad aspects of tumorigenesis and is frequently mutated in a majority of human cancers. We identified a role for mutant forms of p53 in maintaining the malignant, disorganized and invasive, state of breast cancer cells using a three-dimensional (3D) culture model of breast cancer. In this study, we queried a number of pathways/proteins that had previously been shown to be sufficient to effect a phenotypic reversion in 3D culture to search for additional mechanisms by which mutant p53 might contribute to mammary carcinogenesis. Using this approach, we identified β4 integrin as a novel target of mutant p53 in breast cancer cells and demonstrated that stable knockdown of β4 integrin is sufficient to severely blunt the invasive phenotype of breast cancer cells grown in 3D culture. We also demonstrate that inhibition of NF-κB signaling, a downstream mediator of β4 integrin, is sufficient to elicit a phenotypic reversion to acinar-like structures in 3D culture. Furthermore, we show that mutant p53 associates with the promoter of *ITGB4*, the gene encoding β4 integrin, providing a plausible mechanism for its upregulation of *ITGB4* expression. These findings suggest that integrin β4 may be a potential therapeutic target in tumors expressing mutant p53.

INTRODUCTION

p53 is one of the most widely studied proteins in tumor biology and is the most frequently mutated gene in all of human cancer (Petitjean et al., 2007b; Vogelstein et al., 2000). Wild-type p53 is activated in response to a number of cellular stressors including DNA damage, hypoxia and oncogene activation, which leads to cell cycle arrest, apoptosis or senescence, thereby functioning to suppress tumor development (Riley et al., 2008; Vousden and Prives, 2009). *TP53*, the gene encoding p53 protein, is most commonly mutated by single amino acid substitutions (missense mutations) that lead to elevated expression of a mutant form of p53 (Brosh and Rotter, 2009; Levine et al., 1995). While wild-type p53 functions as a tumor suppressor, certain mutant forms of p53 have been shown to exert oncogenic (also termed gain-of-function) effects independent of wild-type p53 (Brosh and Rotter, 2009; Goh et al., 2011; Oren and Rotter, 2010).

Gain-of-function effects of mutant p53 have been shown to impact multiple stages of tumorigenesis including proliferation, inhibition of apoptosis, chemoresistance, invasion, migration and metastasis (Bossi et al., 2006; Dhar et al., 2008; Di Agostino et al., 2006; Lang et al., 2004; Lim et al., 2009; Muller et al., 2009; Olive et al., 2004; Stambolsky et al., 2010). Just as wild-type p53 functions primarily as a transcription factor, many mutant p53 gain-of-function effects are mediated through transcriptional regulation of target genes (Di Agostino et al., 2006; Kim and Deppert, 2007; Matas et al., 2001; Strano et al., 2007; Weisz et al., 2007; Weisz et al., 2004; Yan and Chen, 2010). Interestingly, one emerging theme appears to be that transactivation

targets of mutant p53 are often repression targets of wild-type p53 (Brosh and Rotter, 2009; Di Agostino et al., 2006; Peart and Prives, 2006; Stambolsky et al., 2010).

Integrins are transmembrane receptors, each of which consists of one α and one β subunit, that transmit both chemical and mechanical signals between cells and between the cell and the extracellular matrix (Guo and Giancotti, 2004). Integrin β4, encoded by *ITGB4*, almost exclusively associates with α6 integrin and functions as a receptor for laminins 1, 2, 4 and 5 (Lee et al., 1992). The α6β4 integrin is located on the basal surface of most epithelial tissues where it mediates hemidesmosome formation (Guo and Giancotti, 2004). The importance of this integrin is highlighted by the fact that β4-knock-out mice die a few hours after birth due to respiratory failure, gastrointestinal problems and severe skin blistering (Dowling et al., 1996). The observed phenotype in knock-out mice is remarkably similar to those observed in the inherited human condition, junctional epidermolysis bullosa with pyloric atresia, many cases of which have been shown to be due to mutation of integrin β4 (Dowling et al., 1996; Niessen et al., 1996; Takaoka et al., 1998; van der Neut et al., 1996).

In contrast to other integrins, which have extremely short C-terminal, intracellular domains, the C-terminus of integrin β4 contains a very long cytoplasmic tail that has been shown to impact multiple intracellular signaling pathways (PI3K, Rac1 and RhoA, with NF-κB serving as a common downstream mediator) (Guo and Giancotti, 2004; Guo et al., 2006; Mercurio et al., 2001; Takaoka et al., 1998; Tennenbaum et al., 1996; Weaver et al., 2002). While the regulation of integrin β4 expression is not well understood, it is known to be regulated at both the transcriptional and post-translational levels (Giancotti et al., 1992; Tennenbaum et al., 1996).

The role that integrins play in tumorigenesis is becoming increasingly recognized. Paradoxically, tumors often secrete abundant levels of basement membrane proteins and overexpress β4 integrin, in spite of the fact that they concomitantly express high levels of matrix degrading enzymes (Weaver et al., 2002). In addition, high levels of integrin β4 have been linked to the basal-like subtype of breast cancer and have been shown to have prognostic significance (Lu et al., 2008). A large body of literature has linked the α6β4 integrin to multiple aspects of tumorigenesis including increased survival, angiogenesis and invasion (Lipscomb et al., 2005; Nikolopoulos et al., 2004; Shaw et al., 1997). In fact, integrin β4 was first discovered as a tumor antigen (originally called TSP180 for "Tumor Specific Protein of 180 kDa") expressed at high levels in invasive carcinomas (Giancotti et al., 1992). However, as mentioned in Chapter 1, the role that α6β4 integrin plays in tumorigenesis is still debated.

We had previously found that depletion of endogenous mutant p53 from breast cancer cell lines can dramatically alter their behavior in a three-dimensional culture system by co-opting the SREBP transcription factors and driving flux through the mevalonate pathway. We found that depletion of mutant p53 from MDA-231 cells results in a profound reduction in invasive characteristics, while depletion of mutant p53 from MDA-468 cells is sufficient to elicit a phenotypic reversion to acinar-like structures in 3D culture (Chapter 2). In this study, we have sought additional transcriptional targets of mutant p53 which might contribute to the disrupted morphology of breast cancer cells in 3D culture.

RESULTS

Endogenous mutant p53 regulates integrin β4 expression in breast cancer cells

As reviewed in Chapter 1, a number of pathways and/or proteins have previously been demonstrated to be necessary to maintain the malignant state of breast cancer cells (Table 3.1). We performed a genome-wide expression analysis of MDA-468 breast cancer cells grown in 3D cultures with full or depleted levels of endogenous mutant p53 (Figure 2.3) and queried the genes significantly affected by mutant p53 depletion for those previously shown to produce a phenotypic reversion in tumorigenic breast cells grown in a laminin-rich extracellular matrix.

We examined the mRNA expression of seventeen genes corresponding to fifteen known pathways/proteins demonstrated to be necessary to maintain the disrupted morphology of breast cancer cells in 3D culture, seven of which were significantly affected ($p<0.05$) by mutant p53 depletion. However, only two such genes (*ITGB1* and *ITGB4*), encoding two integrins, were significantly altered in the expected direction to contribute to the phenotypic reversion we observed upon mutant p53 depletion (Table 3.1). Integrin β1 is one of the most well studied integrins in respect to mammary acinar morphogenesis and inhibition of integrin β1 has been shown in multiple reports to revert disrupted tumorigenic breast cells to a more acinar-like morphology (Wang et al., 2002; Wang et al., 1998; Weaver et al., 1997). Integrin β4 is much less studied in relation to mammary tissue architecture disruption, but a number of studies have demonstrated that inhibition of integrin β4 can dramatically alter the morphology of breast cancer cells grown in 3D culture (Dutta and Shaw, 2008; Gabarra et al., 2010; Lipscomb et al., 2005).

We first validated these expression changes using reverse transcription followed by quantitative polymerase chain reaction (qRT-PCR) in three independent experiments of MDA-468.shp53 cells cultured in 3D conditions. While *ITGB1* was significantly downregulated in MDA-468.shp53 cells upon addition of doxycycline, and consequent mutant p53 depletion, this

effect was not observed with mutant p53 knockdown in MDA-231.shp53 cells (Figure 3.1C and 1E). Additionally, we examined integrin β1 levels, the protein encoded by *ITGB1*, upon mutant p53 depletion from MDA-468 cells, but observed only a mild decrease in protein levels (Figure 3.S1B). *ITGB4* mRNA expression was significantly affected by mutant p53 depletion in both a stable pool and stable clone of MDA-468.shp53 cells, as well as in MDA-231.shp53 cells grown in 3D culture (Figure 3.1A, 1C and 1E). Additionally, we examined whether mutant p53 depletion affected nascent transcripts of *ITGB4* by using primers that anneal to a region within intron 3. As mutant p53 depletion also downregulated primary transcripts of *ITGB4*, this regulation occurs as a result of transcription (as opposed to mRNA stability or some later point of regulation) (Figure 3.1C and 1E). Finally, we confirmed that the levels of integrin β4, the protein encoded by *ITGB4*, were also downregulated upon mutant p53 depletion from MDA-468.shp53 and MDA-231.shp53 cells and that this regulation is present in cells grown in both 2D and 3D culture (Figure 3.1B, 1D and 1F). Interestingly, we consistently noted higher protein expression of integrin β4 when cells were grown in 3D compared to 2D culture (Figure 3.1).

Endogenous mutant p53 associates with DNA in the vicinity of transcriptional start sites in the *ITGB4* promoter and within the first intron

Wild-type p53 functions primarily as a transcription factor and we and others have previously demonstrated that mutant p53 can associate with promoter regions to transactivate target genes (Chapter 2) (Stambolsky et al., 2010; Yan and Chen, 2009). The *ITGB4* gene has two alternate transcriptional start sites, which both produce the same protein, but differ in their 5' untranslated region (UTR) (Takaoka et al., 1998). To examine the mechanism by which mutant p53 regulates *ITGB4* expression, we performed chromatin immunoprecipitation followed by

quantitative PCR for a number of regions upstream and downstream of both transcriptional start sites (Figure 3.2A).

We identified two peaks of significant binding by mutant p53 in the vicinity of the *ITGB4* gene, just upstream of each transcriptional start site (TSS_1 and TSS_2). More specifically these were located approximately 300 bp upstream of TSS_1 and approximately 1.8 kb into the first intron and just upstream of TSS_2, with an intervening "valley" (Figure 3.2B and 2C). We observed similar mutant p53 binding in both 2D and 3D culture conditions (Figure 3.2B and 2C, respectively) and this binding was approximately 2-5 fold above binding at a negative site (downstream of the *CDKN1A* gene) and upstream and downstream regions surrounding the *ITGB4* gene and was consistently reduced upon mutant p53 depletion.

Depletion of integrin β4 reduces invasive morphology of MDA-231 breast cancer cells grown in 3D culture

We next examined whether downregulation of integrin β4 following mutant p53 depletion might contribute to the phenotypic effects we observed in 3D culture (Figure 2.1). Using lentiviral transduction, we engineered MDA-231 cells to stably express either a control shRNA or an shRNA targeting integrin β4 in exon 3 using two different hairpin loop structures (shβ4-#1a and shβ4-#1b). When these cells were grown under 3D culture conditions, the parental cells and those infected with a scrambled shRNA lentivirus (Control) exhibited an invasive ("stellate") morphology as previously described (Kenny et al., 2007) and (Chapter 2). However, MDA-231 cells depleted of integrin β4 formed much smaller clusters of cells with almost a complete loss of invasive processes (Figure 3.3A). Nicely, the morphologies of these cell lines tracked with the degree of integrin β4 depletion (Figure 3.3A and 3B). This is in

agreement with previous reports that integrin β4 can promote migration and invasion in a variety of cell lines through either the phosphatidylinositol 3-kinase (PI3K) pathway or the small GTPases (RhoA or Rac1) (Mercurio et al., 2001).

Importantly, this profound reduction in invasive processes closely resembles the phenotypic changes upon mutant p53 depletion from MDA-231 cells (Figure 2.1), but it should be pointed out that these cells do not undergo a complete phenotypic reversion to form acinar-like structures with a hollow lumen upon either mutant p53 or integrin β4 depletion.

NF-κB inhibition is sufficient to induce a phenotypic reversion in MDA-468 breast cancer cells grown in 3D culture

Since we were unable to obtain MDA-468 cells with stable knockdown of integrin β4, we examined whether modulation of downstream signaling pathways might be sufficient to substitute for integrin β4 depletion. While integrin β4 has been shown to influence a number of intracellular signaling pathways, one of the key downstream mediators of many pro-survival effects of integrin β4 is NF-κB (Friedland et al., 2007; Nikolopoulos et al., 2004). As a readout for NF-κB activity downstream of integrin β4, we examined whether canonical NF-κB target genes such as *CXCL1* and *IL8* (Anisowicz et al., 1991; Kang et al., 2007; Kunsch and Rosen, 1993) were affected by mutant p53 depletion. Indeed, *CXCL1* was significantly downregulated upon mutant p53 depletion from both MDA-468.shp53 and MDA-231.shp53 cells grown in 3D culture (Figure 3.4A and 4B) and *IL8* was significantly downregulated in MDA-468.shp53 grown in 3D culture upon mutant p53 depletion (Figure 3.4A).

To examine whether activation of NF-κB was necessary to maintain the malignant, disordered morphology of breast cancer cells in 3D culture, we utilized a previously described

inhibitor of NF-κB (CAY10512) (Heynekamp et al., 2006; Lukiw et al., 2008). MDA-468 breast cancer cells cultured in a laminin-rich ECM in the absence (Control) developed highly disordered, malignant appearing structures as described in Chapter 2. Strikingly however, inhibition of NF-κB activity was sufficient to induce a phenotypic reversion, leading to an increase in the hollow lumen acinar-like population in MDA-468 cells, similar to mutant p53 depletion from these cells (Figure 3.4C and 4D).

***TP53* mutational status and integrin β4 expression may predict patient prognosis**

As presented in Chapter 1, the role of α6β4 integrin in tumorigenesis remains controversial. While most studies report elevated expression and oncogenic activities of integrin β4 (nearly all of which have utilized mutant p53 bearing cell lines), there are now multiple publications detailing tumor suppressive functions of integrin β4. Integrin β4 has been shown to induce apoptosis in a wild-type p53 dependent manner, as well as mediate cell cycle arrest through the p53-target gene, p21 (Bachelder et al., 1999; Clarke et al., 1995; Davis et al., 2001; He et al., 2008). With this in mind, we hypothesized that p53 mutation might function as a molecular switch between the tumor suppressive and oncogenic functions of α6β4 integrin.

While both mutant p53 and integrin β4 have been used as prognostic tools in breast cancer (Lu et al., 2008; Petitjean et al., 2007a), our hypothesis suggested that the combination of p53 status and integrin β4 expression level might serve as a better prognostic indicator in breast cancer than either factor alone. We first examined five human breast cancer patient datasets (totaling 812 patients) to determine whether mutant p53 status correlated with elevated expression of *ITGB4*. Elevated expression in mutant p53 tumors compared to wild-type p53 tumors was only statistically significant in the (ULL) dataset (Langerod et al., 2007) (Figure

3.5A). Since expression data from this dataset fit with our findings using human breast cancer cell lines in 3D culture, we reasoned that this patient population would be the best cohort in which to examine the hypothesis that p53 mutation serves a molecular switch for integrin β4 function.

To do this, we stratified the ULL dataset (80 patients) for *TP53* status (wild-type vs. mutant) and *ITGB4* expression (high vs. low) and found that breast cancer patients with wild-type p53 expressing tumors had a significantly improved disease-free survival time if their tumors concomitantly expressed high levels of β4 integrin ($p<0.01$) (Figure 3.5B, left panel). Interestingly, this trend was reversed in the presence of mutant p53 (Figure 3.5B, right panel), fitting with the notion that p53 mutation might function as molecular switch for integrin β4. It is important to note however that this trend was no longer observed when we examined all five breast cancer datasets pooled together, even when stratified for estrogen receptor (ER) status (Figure 3.5C). This discrepancy may be due to the different patient characteristics, different expression arrays, etc. that were used to generate the different patient datasets, which may also account for the lack of correlation between p53 mutant status and elevated *ITGB4* expression in these patient populations. While we cannot yet conclude that p53 mutation does in fact serve to switch integrin β4 from a tumor suppressor (in the setting of wild-type p53) to an oncogene (in the setting of mutant p53), these data are suggestive that such an effect may occur in at least a subset of human breast cancer patients.

DISCUSSION

In this study, we queried a set of genes, the protein products of which have previously been shown to be necessary to maintain the disordered, malignant state of tumorigenic breast cells in a 3D ECM model for mammary tumorigenesis. We identified two genes, *ITGB1* and *ITGB4*, as being significantly downregulated upon mutant p53 depletion from MDA-468 breast cancer cells grown in 3D culture. We show that at least in the case of *ITGB4*, this regulation occurs at the level of transcription as primary transcripts are also regulated by mutant p53 and endogenous mutant p53 associates with DNA surrounding the *ITGB4* promoter region. This regulation may have important biological consequences as both mutant p53 and integrin β4 have previously been linked to invasion (Adorno et al., 2009; Mercurio et al., 2001; Muller et al., 2009) and we have demonstrated that downregulation of either mutant p53 or integrin β4 is sufficient to impair invasion in a laminin-rich ECM. Additionally, as NF-κB is a key downstream target of integrin β4, we demonstrate that inhibition of this pro-survival factor is sufficient to elicit a phenotypic reversion in 3D culture to structures with acinar-like morphology.

As mentioned earlier, one of the emerging themes in p53 gain-of-function seems to be that many transactivation targets of mutant p53 are repression targets of wild-type p53 and in fact the focus of this investigation, integrin β4, has previously been shown to be a wild-type p53 repression target (Bon et al., 2009). In fact, integrin β4 has also been reported to be a transcriptional target of both p63 and p73 (Bon et al., 2009; Carroll et al., 2006). Whereas wild-type p53 does not physically interact with its family members, mutant p53 has been shown to bind to both p63 and p73 (Gaiddon et al., 2001). However, this interaction usually results in inhibition of p63/p73 activity (Di Agostino et al., 2008; Di Como et al., 1999; Gaiddon et al., 2001; Strano et al., 2002). It is interesting to speculate that perhaps the interaction between

mutant p53 and p63/p73 usually results in inhibition of p63/p73 function, but in certain circumstances might actually exaggerate other functions of p63 or p73.

As mentioned previously, integrin β4 is quite unique within the integrin family due to its very large C-terminal cytoplasmic domain. A truncated, dominant negative, version of integrin β4 (β4-1355T) which can still form hemidesmosomes, but in which has the C-terminal signaling domain is deleted has been previously described (Nikolopoulos et al., 2005). This mutant integrin β4 has been used to separate the adhesive functions from the signaling functions of integrin β4 and expression of this mutant form is sufficient to delay mammary tumorigenesis induced after ErbB2 overexpression in a mouse model of breast cancer, suggesting that integrin β4 is necessary for oncogenic activity of ErbB2 (Guo et al., 2006). It will be very interesting to examine the role of the C-terminal cytoplasmic domain of integrin β4 in the context of mammary tissue architecture.

As noted above, integrin β4 has been shown to impinge upon multiple intracellular signaling pathways, a number of which have been implicated in tumorigenesis (PI3K, Rac1, RhoA and NF-κB) (Guo and Giancotti, 2004; Mercurio et al., 2001; Weaver et al., 2002). While we have demonstrated a particular reliance on NF-κB for disruption of acinar morphogenesis, it will be very interesting to examine additional mediators of integrin β4 signaling in the 3D culture system. Of note, both Rac1 and RhoA must be geranylgeranylated in order to function (Zhang and Casey, 1996). As we have previously demonstrated that geranylgeranylation is critical for the pro-invasive phenotypic effects of mutant p53 in 3D culture, this may provide a link between the findings presented in Chapters 2 and 3. In other words, both integrin β4 and the mevalonate pathway may be necessary to maintain the malignant state due to their effects on Rac1 and/or

RhoA signaling. With that in mind, it will very interesting to examine the effect of Rac1 or RhoA inhibition on breast cancer cells in 3D culture.

Our findings, in at least one cohort of human breast cancer patients, that *TP53* mutation can switch the prognostic impact of *ITGB4* expression from high expression predicting good outcome in wild-type p53 tumors to high expression correlating with a worse prognosis in mutant p53 tumors suggest that p53 mutation may indeed serve as a molecular switch for integrin β4 function. Although our results suggest that prediction models for breast cancer disease recurrence may be enhanced if p53 status is combined with β4 integrin expression, this may only be true for a subset of patients. In addition, while it is not yet possible to firmly conclude that p53 mutation can switch integrin β4 from a tumor suppressing protein to a tumor promoting factor, with many groups pushing for the development of integrin β4 inhibitors as cancer therapeutics (Giancotti, 2007), our findings issue a cautionary note for use of such inhibitors in wild-type p53 expressing tumors, while also suggesting that integrin β4 may be a good therapeutic target in tumors expressing mutant p53.

EXPERIMENTAL PROCEDURES

Plasmids, siRNA, Antibodies and Reagents

Lentiviruses carrying shRNA directed against integrin β4 were cloned using the Gateway cloning system (Invitrogen). p53 was detected using mAb 1801 or DO-1. Anti-integrin β4 (7) and anti-integrin β1 (M-106) antibodies were purchased from Santa Cruz. Anti-phospho-Akt (Thr308) and anti-phospho-p44/42 MAPK (Thr202/Tyr204) (E10) antibodies (Cell Signaling) were kindly provided by Ron Prywes. Anti-Actin (A2066) antibodies and doxycycline (D9891) were purchased from Sigma-Aldrich. CAY10512 (#10009536) was purchased from Cayman Chemicals.

Cell Lines and Generation of Stable Cell Lines

MDA-468 and MDA-231 cells were maintained in DMEM+10% FBS. All cells were maintained at 37^{o}C in 5% CO_2.

Generation of MDA-468.shp53 and MDA-231.shp53 has been described previously (Chapter 2). To induce shRNA expression, cells were treated with 8 μg/ml doxycycline (DOX) for time periods indicated in the figure legends.

MDA-231.shβ4-#1 and MDA-231.shβ4-#2 were generated by producing lentiviruses carrying shRNAs targeting integrin β4, produced in 293T cells and infecting MDA-231 cells. Cells were selected with puromycin to yield stable pools.

Three Dimensional (3D) Culture

Three-dimensional culture was carried out as previously described (Debnath et al., 2003). Briefly, 8-well chamber slides were lined with 50 µl growth factor reduced Matrigel (BD Biosciences). Cells were then seeded at a density of 5,000 cells/well in Assay Medium (DMEM/F12 + 2% Horse Serum + 10 µg/ml Insulin + 0.5 µg/ml Hydrocortisone) containing 2% Matrigel. Cells were refed with Assay Medium containing 2% Matrigel every 4 days. For RNA/protein analysis from 3D cultures, 35 mm plates were lined with 500 µl Matrigel and cells were seeded at a density of 225,000 cells/plate in Assay Medium + 2% Matrigel. Cells were harvested using Cell Recovery Solution (BD Biosciences) according to the manufacturer's instructions.

Immunostaining and Microscopy

Cells were fixed using 2% formaldehyde at room temperature for at least 30 min. Cells were permeabilized for 10 min at 4^{o}C with 0.5% Triton X-100 and subsequently blocked for 1 hr at room temperature with PBS + 0.1% Tween-20 + 0.1% BSA + 10% goat serum. Primary antibodies were incubated with the cultures for 1-2 hr at room temperature, followed by washing, and addition of fluorescently-conjugated secondary antibodies for 40 min at room temperature. Nuclei were counterstained with DRAQ5 (Cell Signaling #4084). Confocal microscopy was conducted using an Olympus IX81 confocal microscope and analyzed using Fluoview software.

Quantitative RT-PCR

RNA was isolated from cells using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. Complementary DNA was transcribed using Qiagen Quantitect reverse transcription kit. Real-time PCR was carried out on an ABI StepOne Plus using SYBR green dye. Transcript levels were assayed in triplicate and normalized to RPL32 mRNA expression. Relative levels

were calculated using the Comparative-Ct Method ($\Delta\Delta C_T$ method). All primers, unless otherwise noted, were designed with Primer Express (Applied Biosystems).

Immunoblotting

Cells were lysed in TEGN Buffer (420 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM EDTA, 10 mM Tris pH 8.0, 0.5 mM PMSF, protease inhibitors [1 μM benzamidine, 3 μg/ml leupeptin, 0.1 μg/ml bacitracin, and 1 μg/ml macroglobulin]) and subjected to SDS-PAGE. Gels were transferred to nitrocellulose membrane and then blocked in 5% milk in PBS. Membranes were incubated with primary antibody for 1 hour at room temperature, followed by three wash steps and a 20 min incubation with IR-conjugated secondary antibodies. Bands were visualized using the Licor Odyssey system.

Quantitative Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) experiments were carried out as previously described (Beckerman et al., 2009). Briefly, MDA-468 cells were treated with 1% formaldehyde prior to lysis in RIPA Buffer and sonication to yield 500 bp fragments. Protein A/G Sepharose beads were conjugated to anti-p53 antibodies (1801/DO-1) which were subsequently used to immunoprecipitate p53 from 1 mg whole cell lysate. Quantitative ChIP was carried out on an ABI StepOne Plus using SYBR green dye. Genomic locations of putative mutant p53 binding sites were determined using UCSC Human Gene Sorter (hg18).

Patient Analysis

One-tailed t-test was performed to assess the significance of the increase in expression level for *TP53* mutated samples to those with wild-type. The alternative hypothesis H_a was expression

level of *TP53* mutated samples is higher than that of wild type samples.

For individual gene, the test was carried out on five breast cancer datasets: FW-MDG (Haakensen et al., 2010; Muggerud et al., 2010), MicMa (Enerly et al., 2011; Wiedswang et al., 2003), ULL (Langerød et al., 2007), DBCG (Kyndi et al., 2009; Myhre et al., 2010; Nielsen et al., 2006) and Miller (Miller et al., 2005) respectively.

Gene annotation mapping

The expression sets were annotated using Entrez gene identities. Genes of interest were mapped to each of the individual sets through Entrez gene IDs. For FW-MDG and MicMa set, the original Agilent probes were mapped to Entrez IDs using BioMart through R library biomaRt (Ensembl release 54/NCBI36 (hg18) human assembly). For Miller set, Affymetrix HG u133a probes were mapped to Entrez IDs by BioMart under the same release. For ULL set, annotations for Stanford 43k cDNA array were retrieved from SMD SOURCE (http://smd.stanford.edu/cgi-bin/source/sourceSearch) under UniGene Build Number 222. Gene identity conversion on DBCG expression set was done using the provided chip annotation file for Applied Biosystems Human Genome Survey Microarray. For the probes shared the same Entrez gene identity, we selected probe(s) with the largest interquartile range (IQR: difference between the third and first quartiles) among the multiple hits. If this still left with more than one hit per Entrez ID, we further averaged the expression values of those probes for each sample.

Datasets

FW-MDG

Two expression sets FW (n = 109) (Muggerud et al., 2010) and MDG (n = 143) (Haakensen et al., 2010) were both from Agilent Whole Human Genome Oligo Microarrays 44k two color

system. In addition, they both are early stage breast cancer cohorts and clinically similar. In this study, we merged the two datasets by gene-median centering on the original probe level. We also excluded normal samples in the MDG set in the study. In total, 139 breast tumors expression profiles with available information on *TP53* status entered the analysis. Among these, 28 samples with mutated *TP53* status and 111 samples with wild-type status.

MicMa

This cohort (Wiedswang et al., 2003) consists of mainly stage I and II breast cancers. mRNA expression profiling was performed on Agilent catalogue design whole human genome 4x44K one color oligo array. Among the 112 tumor samples with available *TP53* status in this sets, 39 samples with mutated *TP53* status and 73 samples with wild-type status.

ULL

This cohort consists of mainly stage I and II breast cancers. Eighty tumors, along with one normal breast tissue sample, were analyzed using Stanford cDNA 43k two color microarrays. We excluded the normal sample in the study, which left 80 tumor samples for the analysis. Among these, 20 samples with mutated *TP53* status and 60 samples with wild-type status.

DBCG

The DBCG series comprise a collection of tumor tissues from 3,083 high-risk Danish breast cancer patients diagnosed in the period 1982–1990 (Kyndi et al., 2009; Myhre et al., 2010; Nielsen et al., 2006). The profiling was carried out on the Applied Biosystems Human Genome Survey one color Microarray. For this study, there were 46 samples with mutated *TP53* status and 104 samples with wild-type status.

Miller

The Miller dataset (Miller et al., 2005) was downloaded from NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) with identifier GSE3494. Data were preprocessed and normalized as described previously (van Vliet et al., 2008). Among the 247 samples, there were 58 samples with mutated *TP53* status and 189 samples with wild type status.

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REFERENCES

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V.*, et al.* (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell *137*, 87-98.

Anisowicz, A., Messineo, M., Lee, S.W., and Sager, R. (1991). An NF-kappa B-like transcription factor mediates IL-1/TNF-alpha induction of gro in human fibroblasts. J Immunol *147*, 520-527.

Bachelder, R.E., Marchetti, A., Falcioni, R., Soddu, S., and Mercurio, A.M. (1999). Activation of p53 function in carcinoma cells by the alpha6beta4 integrin. J Biol Chem *274*, 20733-20737.

Beckerman, R., Donner, A.J., Mattia, M., Peart, M.J., Manley, J.L., Espinosa, J.M., and Prives, C. (2009). A role for Chk1 in blocking transcriptional elongation of p21 RNA during the S-phase checkpoint. Genes Dev *23*, 1364-1377.

Bon, G., Di Carlo, S.E., Folgiero, V., Avetrani, P., Lazzari, C., D'Orazi, G., Brizzi, M.F., Sacchi, A., Soddu, S., Blandino, G.*, et al.* (2009). Negative regulation of beta4 integrin transcription by homeodomain-interacting protein kinase 2 and p53 impairs tumor progression. Cancer Res *69*, 5978-5986.

Bossi, G., Lapi, E., Strano, S., Rinaldo, C., Blandino, G., and Sacchi, A. (2006). Mutant p53 gain of function: reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression. Oncogene *25*, 304-309.

Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer *9*, 701-713.

Carroll, D.K., Carroll, J.S., Leong, C.O., Cheng, F., Brown, M., Mills, A.A., Brugge, J.S., and Ellisen, L.W. (2006). p63 regulates an adhesion programme and cell survival in epithelial cells. Nat Cell Biol *8*, 551-561.

Clarke, A.S., Lotz, M.M., Chao, C., and Mercurio, A.M. (1995). Activation of the p21 pathway of growth arrest and apoptosis by the beta 4 integrin cytoplasmic domain. J Biol Chem *270*, 22673-22676.

Davis, T.L., Cress, A.E., Dalkin, B.L., and Nagle, R.B. (2001). Unique expression pattern of the alpha6beta4 integrin and laminin-5 in human prostate carcinoma. Prostate *46*, 240-248.

Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods *30*, 256-268.

Dhar, G., Banerjee, S., Dhar, K., Tawfik, O., Mayo, M.S., Vanveldhuizen, P.J., and Banerjee, S.K. (2008). Gain of oncogenic function of p53 mutants induces invasive phenotypes in human breast cancer cells by silencing CCN5/WISP-2. Cancer Res *68*, 4580-4587.

Di Agostino, S., Cortese, G., Monti, O., Dell'Orso, S., Sacchi, A., Eisenstein, M., Citro, G., Strano, S., and Blandino, G. (2008). The disruption of the protein complex mutantp53/p73 increases selectively the response of tumor cells to anticancer drugs. Cell Cycle *7*, 3440-3447.

Di Agostino, S., Strano, S., Emiliozzi, V., Zerbini, V., Mottolese, M., Sacchi, A., Blandino, G., and Piaggio, G. (2006). Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer Cell *10*, 191-202.

Di Como, C.J., Gaiddon, C., and Prives, C. (1999). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. Mol Cell Biol *19*, 1438-1449.

Dowling, J., Yu, Q.C., and Fuchs, E. (1996). Beta4 integrin is required for hemidesmosome formation, cell adhesion and cell survival. J Cell Biol *134*, 559-572.

Dutta, U., and Shaw, L.M. (2008). A key tyrosine (Y1494) in the beta4 integrin regulates multiple signaling pathways important for tumor development and progression. Cancer Res *68*, 8779-8787.

Enerly, E., Steinfeld, I., Kleivi, K., Leivonen, S.K., Aure, M.R., Russnes, H.G., Ronneberg, J.A., Johnsen, H., Navon, R., Rodland, E.*, et al.* (2011). miRNA-mRNA Integrated Analysis Reveals Roles for miRNAs in Primary Breast Tumors. PLoS One *6*, e16915.

Friedland, J.C., Lakins, J.N., Kazanietz, M.G., Chernoff, J., Boettiger, D., and Weaver, V.M. (2007). alpha6beta4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-kappaB-dependent resistance to apoptosis in 3D mammary acini. J Cell Sci *120*, 3700-3712.

Gabarra, V., Cho, S., Ramirez, M., Ren, Y., Chen, L.L., Cheung, A., Cao, X., Rennard, R., Unruh, K.R., Graff, C.P.*, et al.* (2010). Antibodies directed to alpha6beta4 highlight the adhesive and signaling functions of the integrin in breast cancer cell lines. Cancer Biol Ther *9*, 437-445.

Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. (2001). A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol Cell Biol *21*, 1874-1887.

Giancotti, F.G. (2007). Targeting integrin beta4 for cancer and anti-angiogenic therapy. Trends Pharmacol Sci *28*, 506-511.

Giancotti, F.G., Stepp, M.A., Suzuki, S., Engvall, E., and Ruoslahti, E. (1992). Proteolytic processing of endogenous and recombinant beta 4 integrin subunit. J Cell Biol *118*, 951-959.

Goh, A.M., Coffill, C.R., and Lane, D.P. (2011). The role of mutant p53 in human cancer. J Pathol *223*, 116-126.

Guo, W., and Giancotti, F.G. (2004). Integrin signalling during tumour progression. Nat Rev Mol Cell Biol *5*, 816-826.

Guo, W., Pylayeva, Y., Pepe, A., Yoshioka, T., Muller, W.J., Inghirami, G., and Giancotti, F.G. (2006). Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. Cell *126*, 489-502.

Haakensen, V.D., Biong, M., Lingjaerde, O.C., Holmen, M.M., Frantzen, J.O., Chen, Y., Navjord, D., Romundstad, L., Luders, T., Bukholm, I.K., *et al.* (2010). Expression levels of uridine 5'-diphospho-glucuronosyltransferase genes in breast tissue from healthy women are associated with mammographic density. Breast Cancer Res *12*, R65.

He, Q., Huang, B., Zhao, J., Zhang, Y., Zhang, S., and Miao, J. (2008). Knockdown of integrin beta4-induced autophagic cell death associated with P53 in A549 lung adenocarcinoma cells. FEBS J *275*, 5725-5732.

Heynekamp, J.J., Weber, W.M., Hunsaker, L.A., Gonzales, A.M., Orlando, R.A., Deck, L.M., and Jagt, D.L. (2006). Substituted trans-stilbenes, including analogues of the natural product resveratrol, inhibit the human tumor necrosis factor alpha-induced activation of transcription factor nuclear factor kappaB. J Med Chem *49*, 7182-7189.

Kang, H.B., Kim, Y.E., Kwon, H.J., Sok, D.E., and Lee, Y. (2007). Enhancement of NF-kappaB expression and activity upon differentiation of human embryonic stem cell line SNUhES3. Stem Cells Dev *16*, 615-623.

Kenny, P.A., Lee, G.Y., Myers, C.A., Neve, R.M., Semeiks, J.R., Spellman, P.T., Lorenz, K., Lee, E.H., Barcellos-Hoff, M.H., Petersen, O.W., *et al.* (2007). The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol *1*, 84-96.

Kim, E., and Deppert, W. (2007). Interactions of mutant p53 with DNA: guilt by association. Oncogene *26*, 2185-2190.

Kunsch, C., and Rosen, C.A. (1993). NF-kappa B subunit-specific regulation of the interleukin-8 promoter. Mol Cell Biol *13*, 6137-6146.

Kyndi, M., Overgaard, M., Nielsen, H.M., Sorensen, F.B., Knudsen, H., and Overgaard, J. (2009). High local recurrence risk is not associated with large survival reduction after postmastectomy radiotherapy in high-risk breast cancer: a subgroup analysis of DBCG 82 b&c. Radiother Oncol *90*, 74-79.

Lang, G.A., Iwakuma, T., Suh, Y.A., Liu, G., Rao, V.A., Parant, J.M., Valentin-Vega, Y.A., Terzian, T., Caldwell, L.C., Strong, L.C., *et al.* (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell *119*, 861-872.

Langerød, A., Zhao, H., Borgan, O., Nesland, J., Bukholm, I., Ikdahl, T., Karesen, R., Borresen-Dale, A., and Jeffrey, S. (2007). TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. Breast Cancer Res *9*, R30.

Langerod, A., Zhao, H., Borgan, O., Nesland, J.M., Bukholm, I.R., Ikdahl, T., Karesen, R., Borresen-Dale, A.L., and Jeffrey, S.S. (2007). TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. Breast Cancer Res *9*, R30.

Lee, E.C., Lotz, M.M., Steele, G.D., Jr., and Mercurio, A.M. (1992). The integrin alpha 6 beta 4 is a laminin receptor. J Cell Biol *117*, 671-678.

Levine, A.J., Wu, M.C., Chang, A., Silver, A., Attiyeh, E.F., Lin, J., and Epstein, C.B. (1995). The spectrum of mutations at the p53 locus. Evidence for tissue-specific mutagenesis, selection of mutant alleles, and a "gain of function" phenotype. Ann N Y Acad Sci *768*, 111-128.

Lim, L.Y., Vidnovic, N., Ellisen, L.W., and Leong, C.O. (2009). Mutant p53 mediates survival of breast cancer cells. Br J Cancer *101*, 1606-1612.

Lipscomb, E.A., Simpson, K.J., Lyle, S.R., Ring, J.E., Dugan, A.S., and Mercurio, A.M. (2005). The alpha6beta4 integrin maintains the survival of human breast carcinoma cells in vivo. Cancer Res *65*, 10970-10976.

Lu, S., Simin, K., Khan, A., and Mercurio, A.M. (2008). Analysis of integrin beta4 expression in human breast cancer: association with basal-like tumors and prognostic significance. Clin Cancer Res *14*, 1050-1058.

Lukiw, W.J., Zhao, Y., and Cui, J.G. (2008). An NF-kappaB-sensitive micro RNA-146a-mediated inflammatory circuit in Alzheimer disease and in stressed human brain cells. J Biol Chem *283*, 31315-31322.

Matas, D., Sigal, A., Stambolsky, P., Milyavsky, M., Weisz, L., Schwartz, D., Goldfinger, N., and Rotter, V. (2001). Integrity of the N-terminal transcription domain of p53 is required for mutant p53 interference with drug-induced apoptosis. EMBO J *20*, 4163-4172.

Mercurio, A.M., Rabinovitz, I., and Shaw, L.M. (2001). The alpha 6 beta 4 integrin and epithelial cell migration. Curr Opin Cell Biol *13*, 541-545.

Miller, L.D., Smeds, J., George, J., Vega, V.B., Vergara, L., Ploner, A., Pawitan, Y., Hall, P., Klaar, S., Liu, E.T.*, et al.* (2005). An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc Natl Acad Sci U S A *102*, 13550-13555.

Muggerud, A.A., Hallett, M., Johnsen, H., Kleivi, K., Zhou, W., Tahmasebpoor, S., Amini, R.M., Botling, J., Borresen-Dale, A.L., Sorlie, T.*, et al.* (2010). Molecular diversity in ductal carcinoma in situ (DCIS) and early invasive breast cancer. Mol Oncol *4*, 357-368.

Muller, P.A., Caswell, P.T., Doyle, B., Iwanicki, M.P., Tan, E.H., Karim, S., Lukashchuk, N., Gillespie, D.A., Ludwig, R.L., Gosselin, P.*, et al.* (2009). Mutant p53 drives invasion by promoting integrin recycling. Cell *139*, 1327-1341.

Myhre, S., Mohammed, H., Tramm, T., Alsner, J., Finak, G., Park, M., Overgaard, J., Borresen-Dale, A.L., Frigessi, A., and Sorlie, T. (2010). In silico ascription of gene expression differences

to tumor and stromal cells in a model to study impact on breast cancer outcome. PLoS One *5*, e14002.

Nielsen, H.M., Overgaard, M., Grau, C., Jensen, A.R., and Overgaard, J. (2006). Study of failure pattern among high-risk breast cancer patients with or without postmastectomy radiotherapy in addition to adjuvant systemic therapy: long-term results from the Danish Breast Cancer Cooperative Group DBCG 82 b and c randomized studies. J Clin Oncol *24*, 2268-2275.

Niessen, C.M., van der Raaij-Helmer, M.H., Hulsman, E.H., van der Neut, R., Jonkman, M.F., and Sonnenberg, A. (1996). Deficiency of the integrin beta 4 subunit in junctional epidermolysis bullosa with pyloric atresia: consequences for hemidesmosome formation and adhesion properties. J Cell Sci *109 (Pt 7)*, 1695-1706.

Nikolopoulos, S.N., Blaikie, P., Yoshioka, T., Guo, W., and Giancotti, F.G. (2004). Integrin beta4 signaling promotes tumor angiogenesis. Cancer Cell *6*, 471-483.

Nikolopoulos, S.N., Blaikie, P., Yoshioka, T., Guo, W., Puri, C., Tacchetti, C., and Giancotti, F.G. (2005). Targeted deletion of the integrin beta4 signaling domain suppresses laminin-5-dependent nuclear entry of mitogen-activated protein kinases and NF-kappaB, causing defects in epidermal growth and migration. Mol Cell Biol *25*, 6090-6102.

Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell *119*, 847-860.

Oren, M., and Rotter, V. (2010). Mutant p53 gain-of-function in cancer. Cold Spring Harb Perspect Biol *2*, a001107.

Peart, M.J., and Prives, C. (2006). Mutant p53 gain of function: the NF-Y connection. Cancer Cell *10*, 173-174.

Petitjean, A., Achatz, M.I., Borresen-Dale, A.L., Hainaut, P., and Olivier, M. (2007a). TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene *26*, 2157-2165.

Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S.V., Hainaut, P., and Olivier, M. (2007b). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat *28*, 622-629.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol *9*, 402-412.

Shaw, L.M., Rabinovitz, I., Wang, H.H., Toker, A., and Mercurio, A.M. (1997). Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. Cell *91*, 949-960.

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., Shiff, I., Kogan, I., Shay, M., Kalo, E.*, et al.* (2010). Modulation of the vitamin D3 response by cancer-associated mutant p53. Cancer Cell *17*, 273-285.

Strano, S., Dell'Orso, S., Di Agostino, S., Fontemaggi, G., Sacchi, A., and Blandino, G. (2007). Mutant p53: an oncogenic transcription factor. Oncogene *26*, 2212-2219.

Strano, S., Fontemaggi, G., Costanzo, A., Rizzo, M.G., Monti, O., Baccarini, A., Del Sal, G., Levrero, M., Sacchi, A., Oren, M.*, et al.* (2002). Physical interaction with human tumor-derived p53 mutants inhibits p63 activities. J Biol Chem *277*, 18817-18826.

Takaoka, A.S., Yamada, T., Gotoh, M., Kanai, Y., Imai, K., and Hirohashi, S. (1998). Cloning and characterization of the human beta4-integrin gene promoter and enhancers. J Biol Chem *273*, 33848-33855.

Tennenbaum, T., Li, L., Belanger, A.J., De Luca, L.M., and Yuspa, S.H. (1996). Selective changes in laminin adhesion and alpha 6 beta 4 integrin regulation are associated with the initial steps in keratinocyte maturation. Cell Growth Differ *7*, 615-628.

van der Neut, R., Krimpenfort, P., Calafat, J., Niessen, C.M., and Sonnenberg, A. (1996). Epithelial detachment due to absence of hemidesmosomes in integrin beta 4 null mice. Nat Genet *13*, 366-369.

van Vliet, M.H., Reyal, F., Horlings, H.M., van de Vijver, M.J., Reinders, M.J., and Wessels, L.F. (2008). Pooling breast cancer datasets has a synergetic effect on classification performance and improves signature stability. BMC Genomics *9*, 375.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Wang, F., Hansen, R.K., Radisky, D., Yoneda, T., Barcellos-Hoff, M.H., Petersen, O.W., Turley, E.A., and Bissell, M.J. (2002). Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. J Natl Cancer Inst *94*, 1494-1503.

Wang, F., Weaver, V.M., Petersen, O.W., Larabell, C.A., Dedhar, S., Briand, P., Lupu, R., and Bissell, M.J. (1998). Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc Natl Acad Sci U S A *95*, 14821-14826.

Weaver, V.M., Lelievre, S., Lakins, J.N., Chrenek, M.A., Jones, J.C., Giancotti, F., Werb, Z., and Bissell, M.J. (2002). beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell *2*, 205-216.

Weaver, V.M., Petersen, O.W., Wang, F., Larabell, C.A., Briand, P., Damsky, C., and Bissell, M.J. (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol *137*, 231-245.

Weisz, L., Oren, M., and Rotter, V. (2007). Transcription regulation by mutant p53. Oncogene *26*, 2202-2211.

Weisz, L., Zalcenstein, A., Stambolsky, P., Cohen, Y., Goldfinger, N., Oren, M., and Rotter, V. (2004). Transactivation of the EGR1 gene contributes to mutant p53 gain of function. Cancer Res *64*, 8318-8327.

Wiedswang, G., Borgen, E., Karesen, R., Kvalheim, G., Nesland, J.M., Qvist, H., Schlichting, E., Sauer, T., Janbu, J., Harbitz, T.*, et al.* (2003). Detection of isolated tumor cells in bone marrow is an independent prognostic factor in breast cancer. J Clin Oncol *21*, 3469-3478.

Yan, W., and Chen, X. (2009). Identification of GRO1 as a critical determinant for mutant p53 gain of function. J Biol Chem *284*, 12178-12187.

Yan, W., and Chen, X. (2010). Characterization of functional domains necessary for mutant p53 gain of function. J Biol Chem *285*, 14229-14238.

Zhang, F.L., and Casey, P.J. (1996). Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem *65*, 241-269.

FIGURE LEGENDS

Figure 3.1 Depletion of mutant p53 from breast cancer cells reduces expression of integrin β4

(A) Integrin β4 mRNA is reduced in a stable pool of MDA-468.shp53 cells. A stable pool of MDA-468.shp53 cells was grown in 3D culture for 8 days in the presence or absence of DOX as indicated to deplete cells of mutant p53. Isolated RNA was reverse transcribed and quantitative PCR was performed for *ITGB4* as described in methods. Data presented as mean ± SD for two independent experiments. *indicates $p<0.05$ from a two-tailed t-test.

(B) Integrin β4 protein is reduced in a stable pool of MDA-468.shp53 cells. A stable pool of MDA-468.shp53 cells was grown in 3D culture as in (A) prior to lysis and immunoblotting. p53 was detected using an anti-p53 antibody (PAb1801). Integrin β4 was detected using an anti-integrin β4 antibody (7). Actin serves as a loading control.

(C) Integrin β4 mRNA is reduced in a stable clone of MDA-468.shp53 cells. A stable clone of MDA-468.shp53 cells was grown in 3D cultures as in (A). Samples were processed and analyzed as in (A) for *ITGB4,* nascent *ITGB4* (primers within intron 3) and *ITGB1*. Data presented as mean ± SD for three independent experiments. **indicates $p<0.005$ from a two-tailed t-test.

(D) Integrin β4 protein is reduced in a stable clone of MDA-468.shp53 cells. A stable clone of MDA-468.shp53 cells was grown in 2D or 3D cultures for 8 days in the presence or absence of DOX as indicated prior to lysis and immunoblotting. p53 was detected using an anti-p53

antibody (PAb1801). Integrin β4 was detected using an anti-integrin β4 antibody (7). Actin serves as a loading control.

(E) Integrin β4 mRNA is reduced in MDA-231.shp53 cells. A stable clone of MDA-231.shp53 cells was grown in 3D cultures as in (A). Samples were processed and analyzed as in (A) for *ITGB4,* nascent *ITGB4* (primers within intron 3) and *ITGB1*. Data presented as mean ± SD for three independent experiments. *indicates $p<0.05$, **indicates $p<0.005$ from a two-tailed t-test.

(F) Integrin β4 protein is reduced in MDA-231.shp53 cells. A stable clone of MDA-231.shp53 cells was grown in 2D or 3D cultures for 8 days in the presence or absence of DOX as indicated prior to lysis and immunoblotting. p53 was detected using an anti-p53 antibody (PAb1801). Integrin β4 was detected using an anti-integrin β4 antibody (7). Actin serves as a loading control.

Figure 3.2 Endogenous mutant p53 associates with the *ITGB4* promoter

(A) Genomic locations of PCR primers are indicated beneath schematic of the *ITGB4* promoter. 5' UTR illustrated as white rectangles, CDS exons illustrated as black rectangles. TSS_1 denotes first transcriptional start site; TSS_2 denotes second transcriptional start site. Genomic locations are numbered in relation to TSS_1 and were located using hg18.

(B) ChIP analysis of mutant p53 on the *ITGB4* promoter. MDA-468.shp53 cells were grown in 2D culture for 8 days in the absence or presence of DOX to deplete p53. Mutant p53 was immunoprecipitated from 1 mg of MDA-468.shp53 lysates using anti-p53 antibodies

(1801/DO1). Parallel samples were processed without antibody (Mock IP, "C") to serve as a negative control. Data is presented as mean ± SD of three independent experiments. Values were normalized to the highest immunoprecipitation signal. **indicates $p<0.01$ compared to all of the following: negative site, +DOX, Mock IP, upstream and downstream sites.

(C) ChIP analysis of mutant p53 on the *ITGB4* promoter. MDA-468.shp53 cells were grown in 3D culture for 8 days in the absence or presence of DOX to deplete p53. Mutant p53 was immunoprecipitated from 1 mg of MDA-468.shp53 lysates using anti-p53 antibodies (1801/DO1). Parallel samples were processed without antibody (Mock IP, "C") to serve as a negative control. Data is presented as mean ± SD of three independent experiments. Values were normalized to the highest immunoprecipitation signal. *indicates $p<0.05$ compared to all of the following: negative site, +DOX, Mock IP, upstream and downstream sites.

Figure 3.3 Depletion of integrin β4 from breast cancer cells reduces invasive phenotype in 3D culture

(A) Depletion of integrin β4 affects MDA-231 cell morphology in 3D cultures. MDA-231 parental (top left panel), expressing a control shRNA (top right panel) or expressing an shRNA targeting integrin β4 (bottom panels) were grown in 3D cultures for 8 days. Representative differential interference contrast (DIC) microscopy images are shown. Scale bar, 200 μm.

(B) Efficient depletion of integrin β4 protein in MDA-231 stable cell lines. MDA-231.shScr (Control), MDA-231.shβ4-#1a or MDA-231.shβ4-#1b cells were grown in 2D culture prior to

lysis and immunoblotting. Integrin β4 was detected using an anti-integrin β4 antibody (7). Actin serves as a loading control.

Figure 3.4 NF-κB inhibition reverts MDA-468 breast cancer cells in 3D culture

(A) NF-κB target genes are significantly downregulated by mutant p53 depletion in MDA-468.shp53 cells. MDA-468.shp53 cells were grown in 3D cultures as in Figure 3.1C. Samples were processed and analyzed for *IL8* and *CXCL1*. Data presented as mean ± SD for three independent experiments. **indicates $p<0.005$ from a two-tailed t-test.

(B) NF-κB target genes are significantly downregulated by mutant p53 depletion in MDA-231.shp53 cells. MDA-231.shp53 cells were grown in 3D cultures as in Figure 3.1E. Samples were processed and analyzed for *IL8* and *CXCL1*. Data presented as mean ± SD for three independent experiments. *indicates $p<0.05$ from a two-tailed t-test.

(C) Inhibition of NF-κB leads to a phenotypic reversion in MDA-468 cells grown in 3D cultures. MDA-468 cells were grown in 3D cultures for 8 days in the presence of DMSO (left panel) or CAY10512 (1 μM) (right panel). Nuclei were stained using DRAQ5 (blue) and analyzed by confocal microscopy. Representative images are shown. Scale bar, 50 μm.

(D) Morphometry of MDA-468 cells treated with NF-κB inhibitor. MDA-468.shp53 cells were grown in 3D cultures as in (C) and structures were analyzed by confocal microscopy for morphological categories (see Figure 2.1C for representative images of categories). Structures

(25-50) were counted for each condition and plotted as a percentage of the population. An average of two experiments is shown.

Figure 3.5 Prognostic Impact of *TP53* Status and *ITGB4* Expression in Breast Cancer

(A) Five human breast cancer patient datasets, FW-MDG, MicMa, ULL, DBCG and Miller were analyzed to determine whether tumors bearing mutant p53 correlated with higher expression of *ITGB4*. Patients were stratified based on *TP53* status (wild-type vs. mutant) and expression levels of *ITGB4* were analyzed. p-value represents the result of a one-sided t-test.

(B) Kaplan-Meier plots measuring Disease-Free Survival using the ULL breast cancer dataset (Langerod et al., 2007) for wild-type p53 tumors (left panel) and mutant p53 tumors (right panel). Red = High *ITGB4* expression; Blue = Low *ITGB4* expression. Period shown in months.

(C) Expression data from five human breast cancer patient datasets, FW-MDG, MicMa, ULL, DBCG and Miller were pooled together to give 664 patients. Patients were stratified based on *TP53* status (wild-type vs. mutant) and expression levels of *ITGB4* (high vs. low) and by estrogen receptor (ER) status (ER+ vs. ER-) and disease-free survival was plotted using a Kaplan-Meier plot. p-value represents the difference between wild-type p53 and mutant p53 tumors from a log-rank test.

Table 3.1 Overlap between expression changes after mutant p53 knockdown and known proteins involved in mammary phenotypic reversions

A literature search was performed to identify proteins and/or pathways modulation of which has been shown to be sufficient to elicit a phenotypic reversion in 3D cultures. Each identified gene was queried against genome-wide expression changes in MDA-468 cells grown in 3D culture following mutant p53 depletion. Changes were considered significant if $p<0.05$.

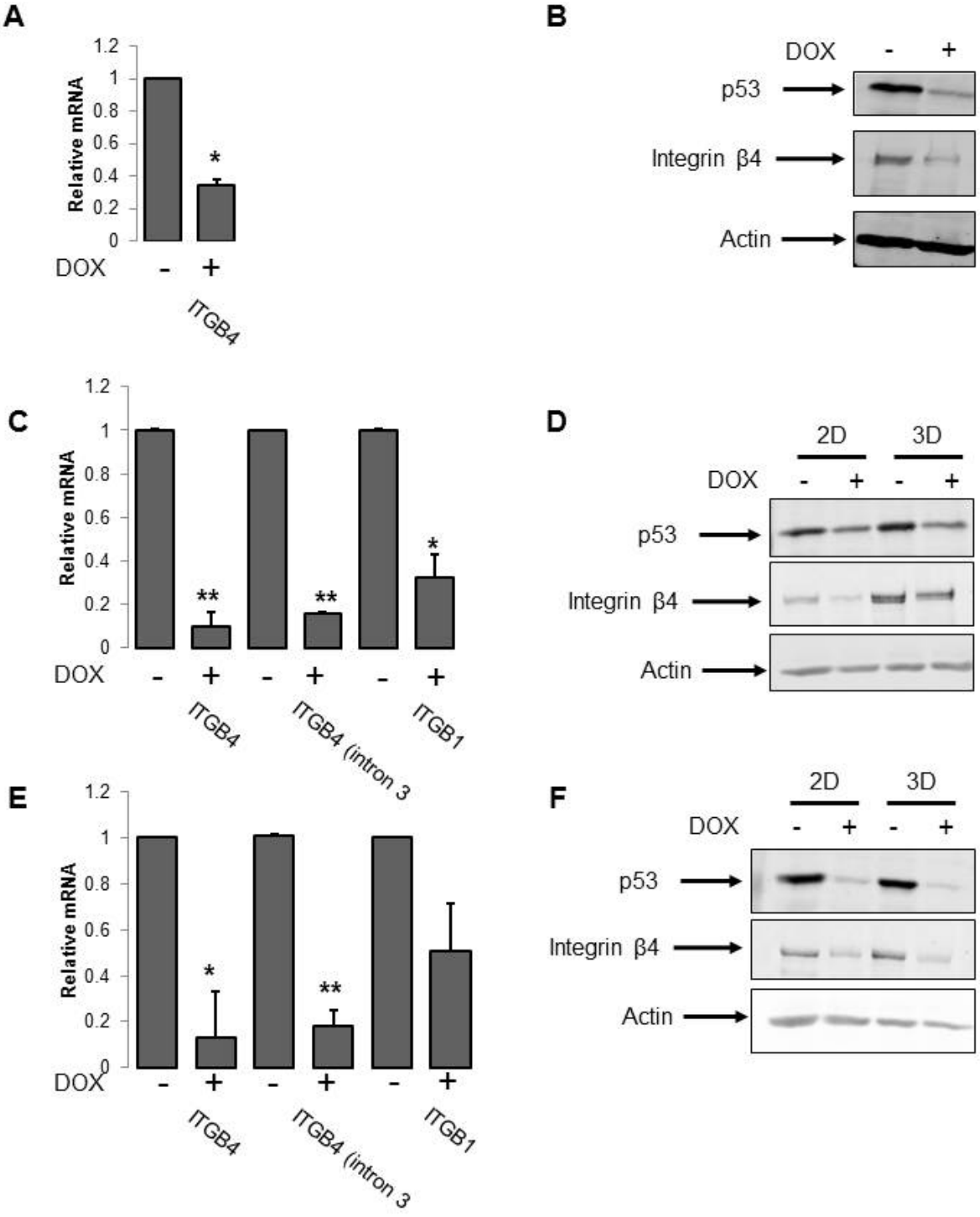
SUPPLEMENTAL FIGURE LEGENDS

Figure 3.S1 ERK-1/2 and PI3K pathways are not affected by mutant p53 depletion from MDA-468 breast cancer cells in 3D culture

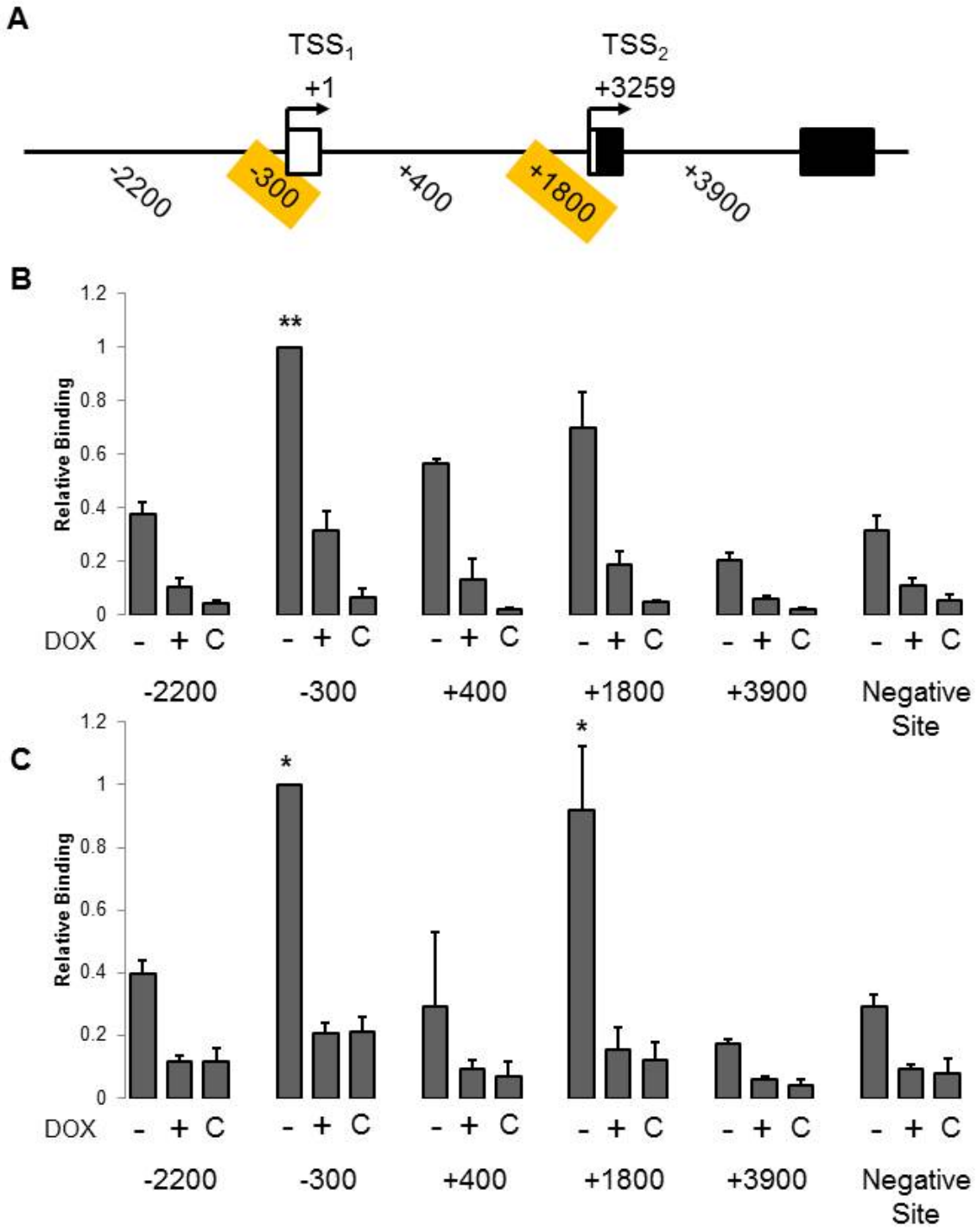
(A) A stable pool of MDA-468.shp53 cells were grown in 3D culture as in Figure 3.1A prior to lysis and immunoblotting. p53 was detected using an anti-p53 antibody (PAb1801). P-Akt was detected using an anti-Phosho-Akt (Thr308) antibody. P-ERK-1/2 was detected using an anti-Phosho-p44/p42 (Thr202/Tyr204) antibody. Actin serves as a loading control.

(B) A stable pool of MDA-468.shp53 cells were grown in 3D culture as in (A) prior to lysis and immunoblotting. p53 was detected using an anti-p53 antibody (PAb1801). Integrin β1 was detected using an anti-Integrin β1 antibody (M-106). Actin serves as a loading control.

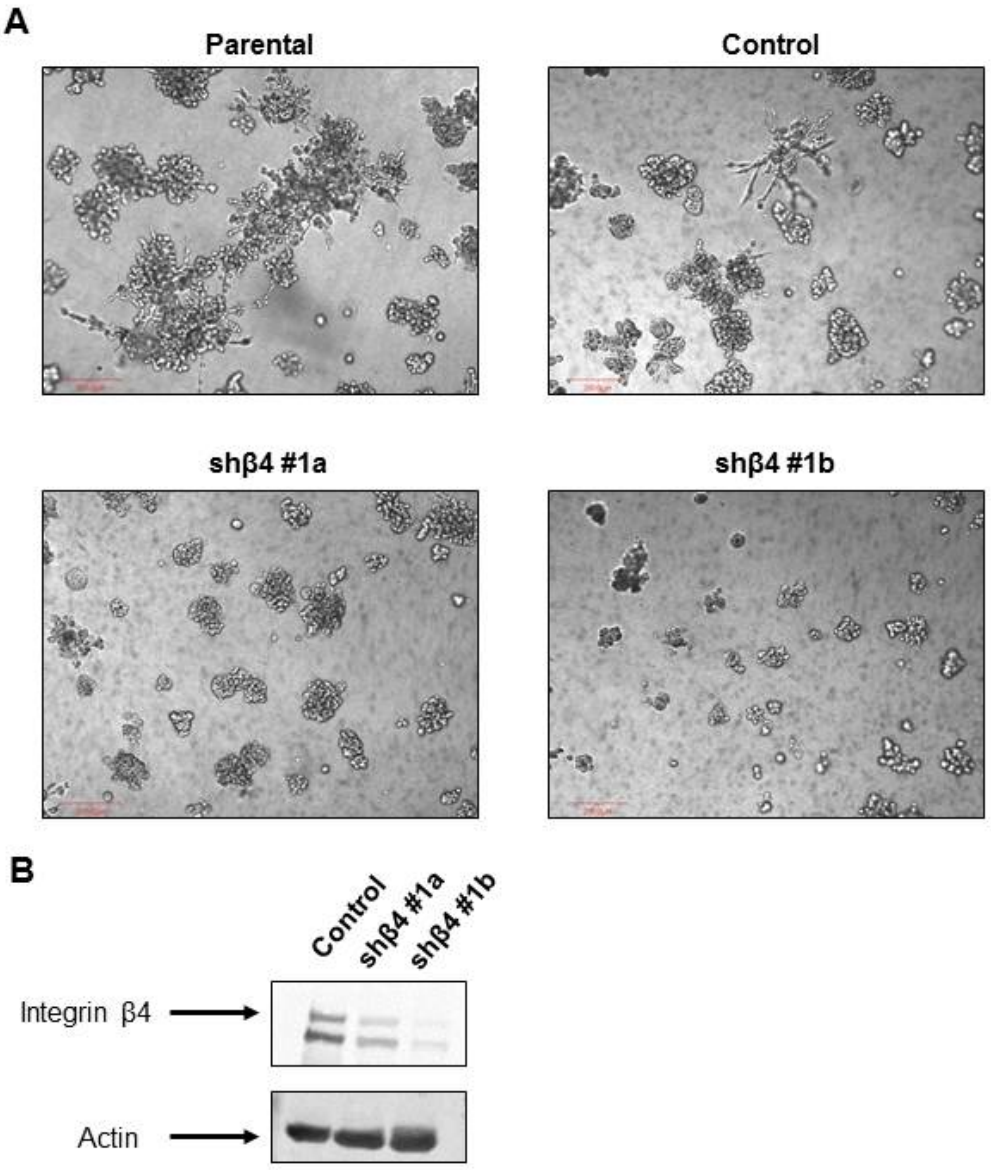
Freed-Pastor et al., Figure 3.1



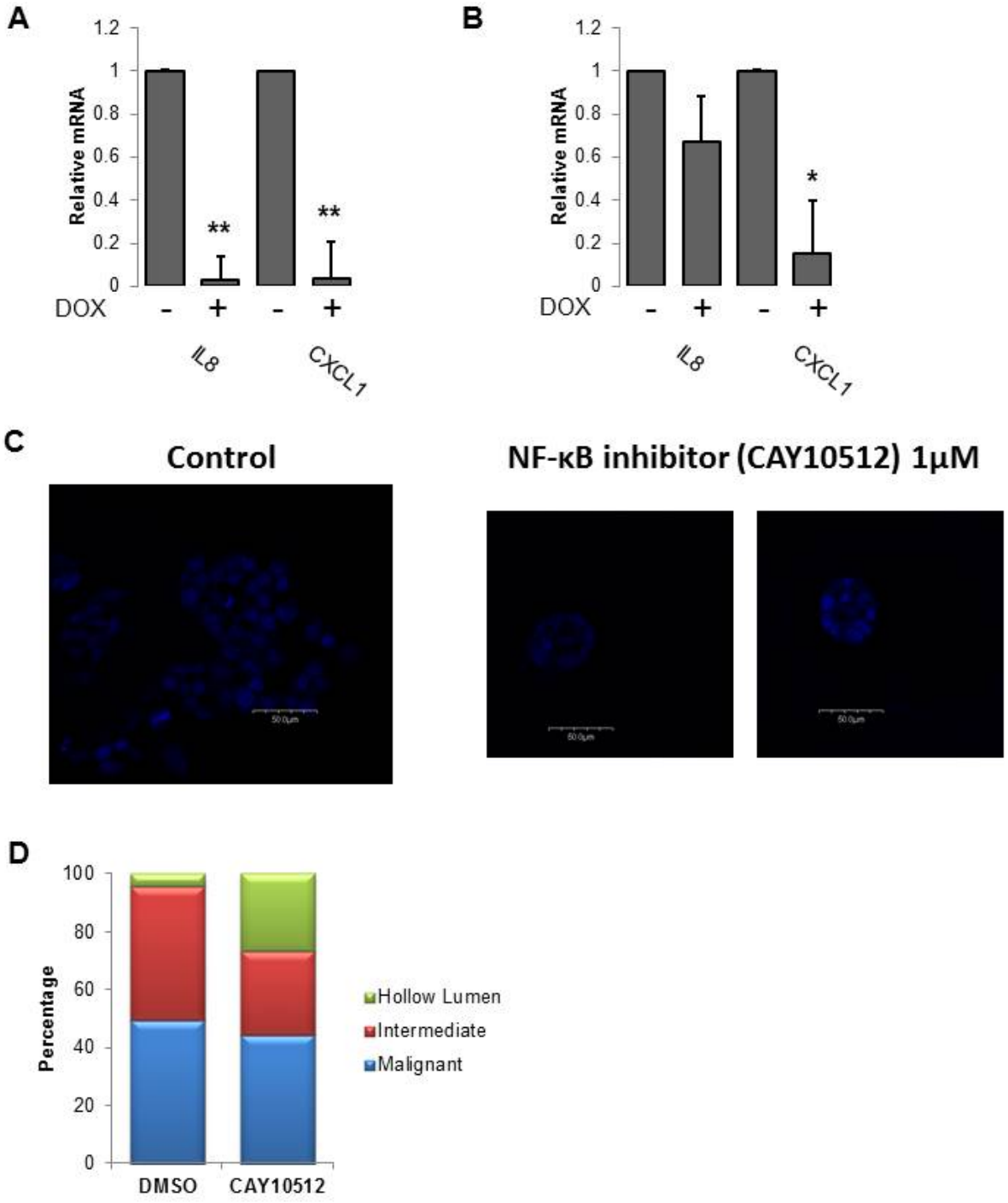
Freed-Pastor et al., Figure 3.2



Freed-Pastor et al., Figure 3.3

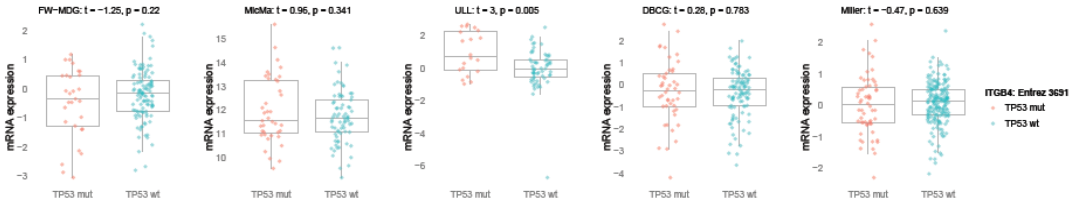


Freed-Pastor et al., Figure 3.4

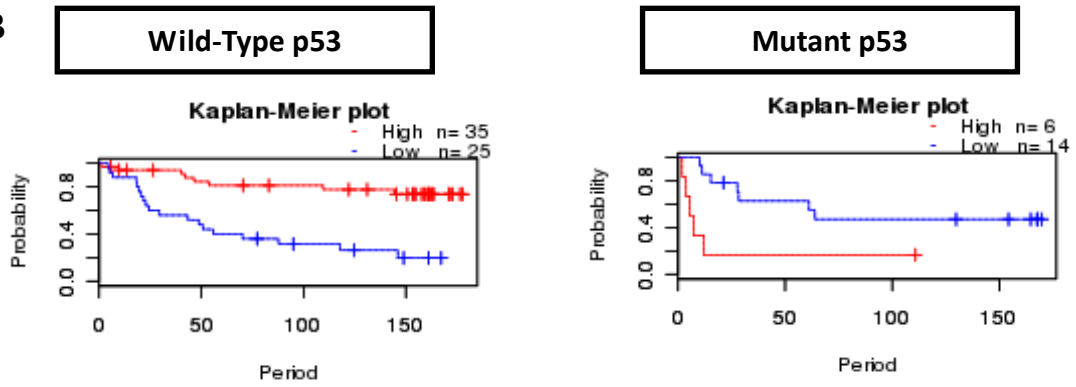


Freed-Pastor et al., Figure 3.5

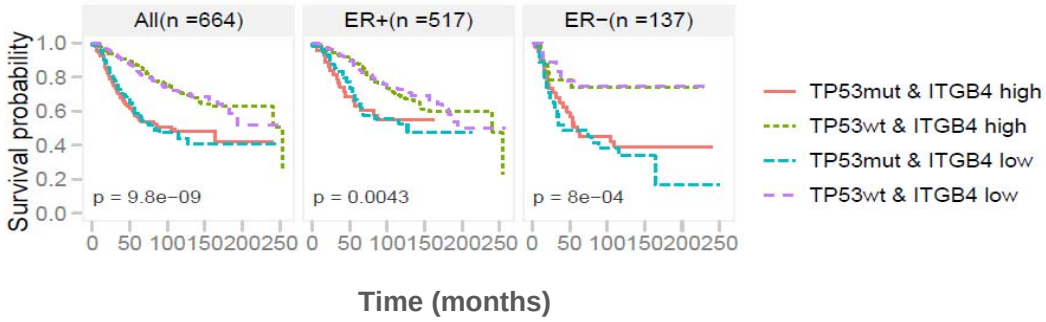
A



B



C



Freed-Pastor et al., Table 3.1

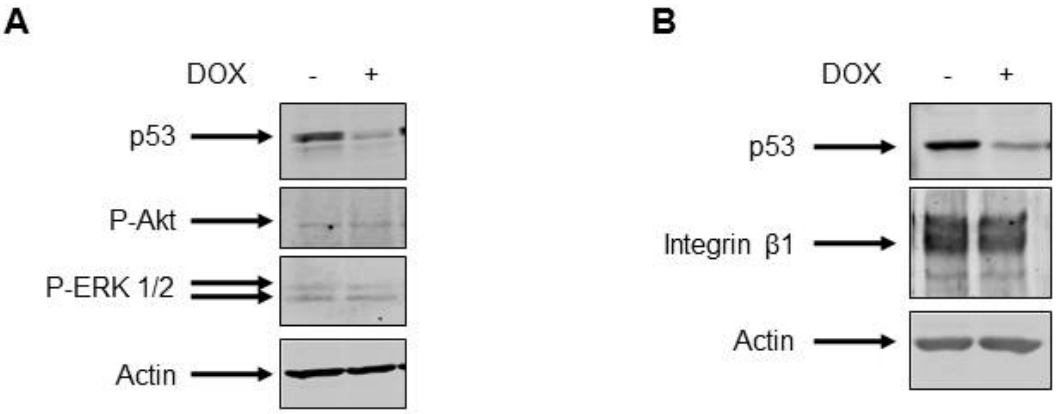
Protein(s)	Gene(s)	Reversion	Mutant p53 regulated[†]
ADAM17	ADAM17	Inhibition (T4-2)	Yes (Up)
AZU-1	TACC2	Re-expression (T4-2)	No
CEACAM1	CEACAM1	Re-expression (MCF7)	Yes (Down)
Dystroglycan	DAG1	Re-expression (T4-2)	Yes (Down)
E-Cadherin	CDH1	Re-expression (multiple)	No
ErbB1	EGFR	Inhibition (T4-2)	Yes (Up)
Fibronectin	FN1	Inhibition (T4-2)	No
HOXD10	HOXD10	Re-expression (MDA-231)	No
Integrin β1	ITGB1	Inhibition (multiple)	**Yes (Down)**
Integrin β4	ITGB4	Inhibition (multiple)	**Yes (Down)**
Integrin α2	ITGA2	Re-expression (Mm5MT)	No
ERK-1/2	MAPK1/MAPK 3	Inhibition (T4-2)	No[**,‡]
MMP-9	MMP9	Inhibition (T4-2)	No
PI3K	PIK3CA	Inhibition (T4-2)	No[**]
Rap1	RAP1A/RAP1B	Inhibition (T4-2)	No

[†]Expression significantly changed in MDA-468 cells upon mutant p53 depletion (p<0.05)

[**]Phosphorylation of ERK-1/2 and Akt unchanged upon mutant p53 depletion from MDA-468 cells (Figure S1A)

[‡]MAPK3, down (p<0.05)

Freed-Pastor et al., Figure 3.S1



Chapter 4

Perspectives and Future Directions

In this dissertation, we have reviewed the importance of the p53 tumor suppressor gene in human cancer and highlighted growing evidence that missense mutations in p53 not only abrogate tumor suppressor function, but can actually endow p53 with oncogenic (gain-of-function) properties. While the gain-of-function hypothesis has existed almost as long as the p53 field, recent years have seen a renewed interest in pro-oncogenic properties of mutant p53 following the seminal finding that mutant p53 knock-in mice exhibit an altered tumor spectrum compared to p53 knock-out mice and mutant p53 confers a metastatic phenotype not observed in p53 null mice (Donehower and Lozano, 2009; Lang et al., 2004; Liu et al., 2000; Olive et al., 2004; Terzian et al., 2008). Over the last five years alone, p53 mutants have been found to actively contribute to tumor proliferation, survival, limitless replication, somatic cell reprogramming, genomic instability, inflammation, migration, invasion, angiogenesis and metastasis (Adorno et al., 2009; Bossi et al., 2006; Brosh and Rotter, 2009; Di Agostino et al., 2006; Girardini et al., 2011; Mizuno et al., 2010; Muller et al., 2009; Oren and Rotter, 2010; Sarig et al., 2010; Song et al., 2007; Stambolsky et al., 2010; Weisz et al., 2007). Amazingly, this places mutant p53 in a central role in tumorigenesis, impacting nine out of the ten (updated) "hallmarks of cancer" proposed by Weinberg and Hanahan (Hanahan and Weinberg, 2011). Interestingly the "hallmark" in which mutant p53 has been least explored to date is "deregulating cellular energetics," which happens to be the focus of Chapter 2.

In Chapter 2, we sought to delineate the phenotypic effects of mutant p53 in breast cancer, particularly as mammary tissue architecture is invariably disrupted during breast

carcinogenesis (Debnath et al., 2003). We describe a novel role for mutant p53 in disrupting acinar morphogenesis and implicate this oncoprotein as a necessary component to maintain the disordered, malignant state of breast cancer cells using a three-dimensional culture model of breast cancer. Specifically, we found that depletion of endogenous mutant p53 from breast cancer cells dramatically reduces invasion or induces a phenotypic reversion in the 3D ECM system, depending on the cellular context. Furthermore, expression of a number of tumor-derived p53 mutants in non-malignant mammary epithelial cells is sufficient to disrupt normal acinar morphogenesis. Intriguingly, both of these functions require an intact transactivation domain suggesting that mutant p53-mediated transcription is responsible for the phenotypic effects on mammary tissue architecture. This is in line with the idea that mutant p53 exerts this activity largely through transcriptional upregulation of seventeen genes in the mevalonate pathway by co-opting the endogenous regulators of this pathway, the sterol regulatory element binding proteins (SREBPs). Mutant p53 is recruited to the promoter regions of a number of SREBP-target genes, which encode enzymes within the mevalonate pathway. Importantly, we also found that supplementation with intermediate metabolites within the mevalonate pathway can substitute for mutant p53 and that inhibition of a number of key enzymes in the mevalonate pathway can profoundly impact the malignant behavior of breast cancer cells grown in a laminin-rich ECM, suggesting that flux through the mevalonate pathway is necessary and sufficient to maintain the malignant state. While it has been demonstrated previously that inhibition and/or downregulation of oncogenic signaling pathways can phenotypically revert breast cancer cells grown in 3D culture (Beliveau et al., 2010; Bissell et al., 2005; Wang et al., 1998; Weaver et al., 1997), we show here that downregulation of mutant p53 and/or inhibition of the mevalonate pathway can function in this capacity. From this work, we posit that tumors bearing mutant p53

evolve to become highly reliant on metabolic flux through the mevalonate pathway. As a number of well-tolerated inhibitors targeting the mevalonate pathway are commonly used in the clinic, this work may offer a novel, and much needed, therapeutic option for tumors bearing mutant p53. In addition to the potential implications of this work, one can envision many avenues in which to follow up on these findings, some of which will be outlined below.

Further Exploration of Mutant p53 in 3D Culture

One interesting avenue might be to examine which mutants of p53 can affect mammary tissue architecture and what aspects of p53 are responsible for these phenotypic effects. We have already demonstrated that an shRNA-resistant version of mutant p53 (R273H) can prevent the phenotypic reversion observed in 3D culture, while a mutant p53 protein that lacks functional transactivation subdomains (L22Q/W23S/W53Q/F54S "mTAD") (Lin et al., 1994; Zhu et al., 1998) cannot rescue this phenotypic reversion (Figure 2.2). To examine whether other tumor-derived mutants of p53 can substitute for endogenous p53-R273H in maintaining the malignant, disordered state of MDA-468 breast cancer cells in 3D culture, we engineered MDA-468.shp53 cells to express a number of other shRNA-resistant p53 mutants, along with their respective transactivation-deficient versions. Interestingly, while p53-R273H, -G245S and -R248W can partially rescue the effect of depleting MDA-468 cells of endogenous mutant p53, p53-R175H and all mTAD versions do not have a similar effect on MDA-468 morphology in 3D culture (Figure 4.1) although this must be interpreted with caution, as expression of p53-R175H is lower than other mutants (data not shown).

Similar to the approach taken with mutation of the transactivation domains of mutant p53, it is possible to create altered versions of mutant p53 that would help to dissect additional

aspects of p53 biology. For example, mutation of leucine at position 344 has previously been shown to modulate oligomerization status, L344A prevents tetramerization, but allows dimer formation, while L344P abolishes all oligomerization (Davison et al., 1998). If placed in the context of a tumor-derived mutation, this would provide insight into whether mutants of p53 need to oligomerize in order to exert their gain-of-function effects. One can also probe whether the subcellular localization can impact mutant p53 gain-of-function by introducing a second point mutation (K305N) into a tumor-derived mutant p53, which would prevent nuclear translocation by blocking p53 binding to the nuclear localization signal (NLS) receptor (Liang and Clarke, 1999). A subset of tumor-derived mutant p53 proteins have recently been demonstrated to aggregate, a property which can be suppressed by mutation (I254R) within the "aggregation signal" (Xu et al., 2011). It will be interesting to introduce this mutation into the context of tumor-derived p53 mutants to examine whether aggregation is involved in the gain-of-function effects of mutant p53 on mammary tissue architecture. Finally, as the C-terminus of mutant p53 has been shown in some circumstances to be necessary for gain-of-function effects and in other circumstances to play an inhibitory role (Muller et al., 2009; Yan and Chen, 2010), it will be interesting to examine what effect, if any, deletion of this region has in terms of the behavior of breast cancer cells in a laminin-rich ECM.

Additionally, to explore how pervasive the phenomenon that breast cancer cells become reliant on expression of mutant forms of p53 and the mevalonate pathway, it will be interesting to engineer a larger panel of breast cancer cells to express shRNA targeting endogenous mutant p53 and examine their morphology in 3D culture as well as the response of the mevalonate pathway genes to mutant p53 depletion. One of the large unanswered questions from this work is which tumor-derived mutants of p53 can function to upregulate the mevalonate pathway

genes. We have demonstrated that an endogenous mutant p53 (R273H) can upregulate seventeen genes in the mevalonate pathway, as well as a number of other SREBP-target genes. Additionally, we show that a separate endogenous mutant p53 (R280K) can upregulate at least a subset of these SREBP target genes. However, it will be interesting to examine if we can find appropriate cellular conditions under which exogenous mutant p53 can upregulate these genes as this will allow one to investigate a large panel of tumor-derived p53 mutants for their ability to regulate the expression of these genes. Additionally, if these conditions can be readily identified, this will allow one to further explore the mechanism by which tumor-derived p53 mutants upregulate these genes by utilizing many of the altered versions of p53 described above.

Global Analysis of Mutant p53 DNA Binding

During the course of dissecting the effect of mutant p53 in 3D culture, we also performed chromatin immunoprecipitation for mutant p53 followed by massively parallel sequencing (ChIP-Seq) to identify potential mutant p53 binding sites in the genome following mutant p53 depletion from MDA-468.shp53 breast cancer cells grown in 3D culture. After mapping reads to the human genome using Bowtie, we employed model-based analysis of ChIP-Seq (MACS) (Zhang et al., 2008) to identify peaks (potential mutant p53 binding sites). We identified 11,072 peaks with p-value $\leq 10^{-6}$ and fold enrichment ≥ 5 compared to "Mock IP (IgG)" control samples or samples from MDA-468.shp53 cells cultured in the presence of DOX ("+DOX") to deplete endogenous mutant p53 and subjected to ChIP-Seq.

We next identified 2,386 genes with at least one putative mutant p53 binding sites. To assign peaks to genes, we focused on significant peaks that were located in the -3,000 bp to +1,000 bp region surrounding an annotated transcription start site (TSS). Since we had

previously found that mutant p53 is responsible for controlling the expression of a number of sterol biosynthesis genes in human breast cancer cells (Chapter 2), we examined our ChIP-Seq results to see if any mevalonate pathway genes were identified using this method. Indeed, sterol biosynthesis genes were significantly ($p<0.01$) overrepresented in our ChIP-Seq results (Figure 4.2) supporting our original finding that mutant p53 associates with the promoters of a number of mevalonate pathway genes (Figure 2.6). In fact, one of our collaborators recently performed ChIP-Seq for SREBP-1 and SREBP-2 in the human hepatocellular carcinoma cell line HepG2. We plan to compare our ChIP-Seq data with these results to expand upon our findings that link SREBP and mutant p53 in breast cancer. Likewise, as we have demonstrated that Fatostatin, a small molecule that prevents SREBP activation, has profound phenotypic effects on a number of mutant p53 bearing breast cancer cell lines in 3D culture, it will very interesting to perform genome-wide expression analyses on breast cancer cells grown in 3D culture untreated or treated with Fatostatin and compare these with expression changes identified after mutant p53 depletion from these same cells. In addition, it could be interesting to perform a second mutant p53 ChIP-Seq following Fatostatin treatment of MDA-468 breast cancer cells grown in 3D culture to determine what portion of mutant p53 binding events require the SREBP transcription factors. These should provide further insight into the connection between SREBP and mutant p53.

In terms of additional analyses on the existing ChIP-Seq, we first plan to investigate the question: where exactly does mutant p53 tend to bind (i.e. promoters, intragenic regions, intergenic regions, etc.)? Our quantitative ChIP results presented in Chapters 2 and 3 suggest that mutant p53 may have a low level of promiscuous binding with DNA, as even "negative" regions showed a decrease in binding upon mutant p53 depletion. While our initial assignment of potential p53 target sites biased the analysis to a 4 kilobase region surrounding all annotated

transcriptional start sites, it will be of interest to take an unbiased approach for all peaks identified by MACS and examine where mutant p53 tends to bind. We also plan to compare ChIP-Seq results with expression microarray data to examine which binding events actually lead to changes in gene expression. This will be particularly informative since these analyses were performed on MDA-468.shp53 cells grown in parallel in 3D culture and thus may offer valuable information about the role of mutant p53 in 3D culture. As a part of this analysis, it will be interesting to examine how many of these genes are upregulated versus downregulated upon mutant p53 depletion from breast cancer cells to determine whether mutant p53 functions as a transcriptional activator or repressor for given genes. In addition, we plan to perform Gene Ontology (GO) analysis on targets identified by ChIP-Seq, similar to the analysis performed in Figure 2.3, to examine the pathways/processes that mutant p53 is directly regulating.

It may also be interesting to compare putative mutant p53 binding sites identified by ChIP-Seq with previously described genome-wide analyses of wild-type p53 binding (Botcheva et al., 2011; Smeenk et al., 2011; Wei et al., 2006). As wild-type p53 has been demonstrated to have a preference for regions enriched in CpG islands in non-tumorigenic cells, it will be interesting to examine whether mutant p53 global binding displays a similar pattern in breast cancer cells (Botcheva et al., 2011; Freed-Pastor, 2011). Additionally, it may be of interest to perform ChIP-Seq analyses for different histone modifications using MDA-468 breast cancer cells in 3D culture and then compare these patterns to the global pattern of mutant p53 binding. To date there is no "mutant p53 response element," so perhaps, in addition to recruitment through other transcription factors, mutant forms of p53 are recruited to certain promoters based on their chromatin landscape.

As introduced above, one of the more common mechanisms for mutant p53-mediated transcriptional regulation appears to be that mutant forms of p53 can interact with other sequence-specific transcription factors. This allows mutant p53 to be recruited to the cognate binding site of the other cellular transcription factor and either strengthen or dampen the target response. We plan to identify transcription factor response elements that are over-represented in our mutant p53 ChIP-Seq. This method has been previously utilized using a mutant p53 ChIP-on-chip from SK-BR-3 cells, which identified the ability of p53-R175H to interact with and regulate vitamin D receptor (VDR) genes (Stambolsky et al., 2010).

Nearly all studies report that tumor-derived mutants of p53 have lost sequence-specificity, but in general this is meant that they can no longer recognize canonical wild-type p53 response elements. We plan to perform a motif analysis to search for sequences that are over-represented in our mutant p53 ChIP-Seq to examine if p53-R273H may have a preference for certain sequence motifs. These may be similar to or distinct from the wild-type p53 response element. We recognize that interpretation of this analysis may be difficult as it will not be possible to discriminate which motifs mutant p53 is binding to directly, as opposed to those that it is binding to through a separate transcription factor. However, as will be described below, we plan to pursue this question in a combined approach with *in vitro* studies guiding the identification of putative sequence-specific binding sites. This second approach will be described below.

Statins: A new therapy for mutant p53 tumors?

Our findings that tumor-derived p53 mutants upregulate multiple enzymes in the mevalonate pathway and inhibitors targeting a number of these enzymes have dramatic effects

on breast cancer cell morphology in 3D culture, with no effect on wild-type p53 expressing MCF10A cells, suggest that tumors bearing mutant p53 may be more sensitive to inhibition of the mevalonate pathway compared to wild-type p53 expressing cells. However, while we have very strong evidence that inhibition of the mevalonate profoundly affects mutant p53 breast cancer cells, further studies are warranted to examine the relative sensitivity to inhibition of the mevalonate pathway between wild-type and mutant p53 tumors. In fact, one of the more exciting avenues for future research will be to expand upon this finding. It will be interesting to examine a large panel of breast cancer cell lines (or perhaps primary breast tumor samples) for their sensitivity to inhibition of the mevalonate pathway. Of particular interest will be to evaluate inhibition of HMG-CoA reductase using statins, as these drugs are FDA-approved and well-tolerated, and then correlate the relative sensitivities to p53 mutational status. Ideally, this type of study would include cell lines (or primary tumor samples) grown in 3D culture in the presence or absence of a statin, as well as parallel samples xenografted into immunocompromised mice and then untreated or treated with a statin. A study of this kind that included sufficient numbers of both wild-type and mutant p53 samples would add greatly to our understanding of the connection between mutant p53 tumors and statins.

In addition to xenograft experiments using human cancer cells implanted into immunocompromised mice, it will also be interesting to investigate the effect of statins in mouse models of cancer. One such study might evaluate the effect of statins using knock-in mutant p53 mice compared to p53 knock-out mice (Donehower et al., 1992; Lang et al., 2004; Olive et al., 2004). Alternatively, carcinogen-induced tumor models may offer another potential avenue through which to investigate this relationship (Russo and Russo, 1996; Sukumar et al., 1995). For example, to examine the chemopreventive role of statins in mutant p53 tumors, rats either

receiving a placebo or a statin could be treated with 7,12-Dimethylbenz(a)anthracene (DMBA) as a mutagen and resulting tumors could subsequently be analyzed for p53 mutations. If our hypothesis is correct, that mutant p53 tumors are more sensitive to inhibition of the mevalonate pathway, one would expect fewer mutant p53 tumors to arise in statin-treated rats than in the placebo group.

Additionally, it will be very exciting to evaluate mutation of p53 as a potential modifier of statin treatment in clinical studies on breast cancer as well as potentially other tumor types. There are a large number of epidemiologic studies which have investigated the effect of statins in breast cancer and a portion of these report that they included tissue samples for each patient's tumor (Ahern et al., 2011; Fagherazzi et al., 2010). Similar to the pre-clinical studies, we would expect fewer mutant p53 tumors to arise in statin-treated patients than in non-statin-treated patients if our hypothesis is correct. Perhaps the quickest and easiest assay would be to perform p53 immunohistochemical (IHC) staining on tumor sections as a surrogate for p53 mutation; however, this method introduces an unacceptable loss of specificity and sensitivity (Alsner et al., 2008; Bartek et al., 1990), thus we strongly advocate for full sequencing of the *TP53* locus for these analyses. Importantly, it is relatively straightforward to sequence *TP53* from fresh frozen or formalin-fixed, paraffin-embedded (FFPE) tumor sections (Chrisanthar et al., 2011; Lumachi et al., 2009).

Wild-type p53, SREBPs and the Mevalonate Pathway

As mentioned in previous chapters, one of the emerging paradigms in the mutant p53 field is that mutant p53 gain-of-function effects tend to be diametrically opposed to the effects exerted by wild-type p53. For example, wild-type p53 counteracts the activity of NF-Y, Sp1,

Ets-1 and VDR, while mutant p53 enhances the functions of these same proteins (Bargonetti et al., 1997; Brosh and Rotter, 2009; Chicas et al., 2000; Di Agostino et al., 2006; Peart and Prives, 2006; Sampath et al., 2001; Stambolsky et al., 2010). In Chapter 2, we described a novel role for tumor-derived p53 mutants in promoting aberrant activation of the mevalonate pathway through the SREBP transcription factors. An interesting question arises as to what might be the effect (if any) of wild-type p53 on this pathway.

To begin to address this question, we first employed hepatocellular carcinoma cell lines treated with different stimuli to activate wild-type p53. We chose cells derived from liver tissue to investigate potential repression of this pathway since liver cells express high levels of the mevalonate pathway genes (Seo et al., 2009; Seo et al., 2011). The first compound utilized, 5-Fluorouracil (5-FU), is a commonly used chemotherapeutic in breast and colorectal carcinomas (Sun et al., 2007). Once inside cells 5-FU, a pyrimidine analog, is quickly converted to 5-fluoro-deoxyuridine triphosphate (5-fluro-dUTP) and 5-fluoro-deoxyuridine monophosphate (5-fluro-dUMP), which binds to and inhibits the activity of thymidylate synthase, the enzyme responsible for *de novo* synthesis of deoxythymidine monophosphate (dTMP). This results in a change in the deoxynucleotide pool, effectively shutting down new DNA synthesis and resulting in DNA damage. In addition, 5-FU (after being converted to 5-fluoro-dUTP) can be incorporated into ribosomal RNA (rRNA) leading to a halt in ribosome biosynthesis and releasing ribosomal proteins such as L5 and L11 from the nucleolus, which can bind to and inhibit Mdm2 (Sun et al., 2007). Both of these mechanisms have been credited for the activation of wild-type p53 in response to 5-FU treatment, although the mechanism is still not firmly established. The second compound used in this study was Nutlin-3a, a small molecule that disrupts the p53-Mdm2 complex leading to stabilization of both proteins, which was described in Chapter 1.

To examine if wild-type p53 has any effect on the mevalonate pathway, SK-Hep1 and HepG2 cells, both of which harbor wild-type p53, were treated with 5-FU and Nutlin-3a to activate p53 and the mRNA expression of mevalonate pathway genes was assessed. We observed a downregulation upon treatment with both compounds in SK-Hep1 (Figure 4.3A) and HepG2 cells (data not shown), although in general the effects of 5-FU were more pronounced. However, it is important to note that 5-FU can exert many p53-independent effects. To address this, we used RNAi to deplete cells of wild-type p53 concomitantly treated with 5-FU. While the downregulation of mevalonate pathway genes was blunted in the setting of depleted p53, there is most likely an additional p53-independent component to the downregulation (data not shown).

To circumvent the p53-independent effects of 5-FU treatment, we exploited another hepatocellular carcinoma cell line (Hep3B-4Bv), which had been engineered to stably express a temperature-sensitive mutant p53. This cell line expresses a mutant of p53 (p53-A135V) that is structurally unfolded at 37^{o}C, but which re-folds to a wild-type conformation when the temperature is lowered to 32^{o}C (Friedman et al., 1997). We cultured Hep3B-4Bv cells at 37^{o}C or 32^{o}C for 48 hours and assessed mRNA expression of the mevalonate pathway genes. Remarkably, this shift in temperature was sufficient to significantly downregulate all seventeen mevalonate pathway genes tested (Figure 4.3B). Importantly, while these results suggest that wild-type p53 may suppress the mevalonate pathway genes, we cannot yet rule out the possibilities that temperature alone might have an effect or that the mutant p53 (A135V) in these cells might be activating the mevalonate pathway genes and thus we are observing a loss of activation, as opposed to active repression by wild-type p53.

While this is a fascinating area of research, there is still much work to confirm that wild-type p53 represses expression of the mevalonate pathway genes. We plan to expand upon the

initial finding, that mevalonate pathway genes are downregulated upon 5-FU and Nutlin-3a treatment, by examining a larger panel of cell lines with a larger panel of chemotherapeutic drugs. In addition to testing the p53-dependence of this effect, it will also be interesting to examine what role p21 might play as this p53-target gene has been linked to many p53 repression targets (Barsotti and Prives, 2010). In the context of the temperature-sensitive mutant p53, we plan to investigate whether wild-type p53 is actively repressing the mevalonate pathway genes or whether this is simply an alleviation of transactivation. We will use RNAi to deplete p53 at either 37^{o}C or 32^{o}C and examine the expression of the mevalonate pathway genes. If in fact we are observing active repression by wild-type p53, we should only see an effect at 32^{o}C, while the opposite will be true if p53-A135V is actively upregulating these genes.

In addition, it will be potentially very interesting to examine the effect of p53 family members, p63 and p73, on SREBP-target genes, with a specific focus on the mevalonate pathway. There is some evidence to suggest that these proteins may impact upon SREBP-mediated transcription as both p63 and p73 have been shown to upregulate the expression of *FASN*, which encodes fatty acid synthase (Sabbisetti et al., 2009). Furthermore, similar to p53, p73 is induced following DNA damage (Urist et al., 2004) and thus may account for a portion of the p53-independent effects noted after 5-FU treatment of HepG2 and SK-Hep1 cell lines. We also plan to examine whether wild-type p53 or its family members can physically interact with the SREBP family of transcription factors and whether wild-type p53 or its family members can interact with the promoter regions of the mevalonate pathway genes as assessed by chromatin immunoprecipitation.

Although we cannot yet conclude that wild-type p53 is repressing the sterol biosynthesis pathway, it is interesting to speculate about the potential implications if this regulation does turn

out to exist. For example, a connection between p53, the mevalonate pathway and the mammary gland may be re-visited. Wild-type p53 has been found to be excluded from the nucleus during normal lactation (Moll et al., 1992). Milk is rich in both cholesterol and fatty acids and SREBPs have been documented to play a role in normal lactation (Rodriguez-Cruz et al., 2006; Rudolph et al., 2007). If wild-type p53 does in fact repress mevalonate pathway genes, it is tempting to speculate that p53 is excluded from the nucleus during lactation in order for efficient milk production to occur. As described in Chapter 1, Eph4 cells, a non-malignant murine mammary epithelial cell line that harbors wild-type p53, can be stimulated to induce lactation-specific genes (β-casein, etc.) by growth in a laminin-rich ECM and treatment with lactogenic hormones. In fact, we have grown Eph4 cells in 3D culture treated with Prolactin (PRL) a lactogenic hormone and verified that β-casein mRNA expression is dramatically induced as a read-out for milk synthesis (data not shown). It might be interesting to examine this hypothesis by culturing Eph4 cells in a 3D laminin-rich ECM treated with Prolactin and immunostain these structures for p53 to confirm that it is excluded from the nucleus during lactation. In addition to β-casein expression, one would want to confirm that the mevalonate pathway genes are upregulated in response to lactogenic hormones. If both of these held true for Eph4 murine mammary epithelial cells stimulated to lactate in 3D culture, one could explore the hypothesis that exclusion of wild-type p53 is necessary for lactation to occur by culturing these cells in the presence of leptomycin B (LMB), which has been shown to prevent nuclear export of p53. However, LMB is not specific to p53, therefore one could also engineer these cells to express a version of p53 in which the nuclear export signal (NES) had been mutated (Stommel et al., 1999).

Integrin β4

In Chapter 3, we examined alternate mechanisms by which mutant p53 might contribute to maintenance of the malignant state. By querying a set of genes, the protein products of which had previously been shown to be necessary to maintain the disordered, malignant state of tumorigenic breast cells in the 3D ECM model system, we identified two genes, *ITGB1* and *ITGB4*, that were significantly downregulated upon mutant p53 depletion from breast cancer cells grown in 3D culture in the expected direction. We demonstrate that endogenous mutant p53 directly regulates integrin β4, as it associates with DNA surrounding the *ITGB4* promoter region. We also show that downregulation of either mutant p53 or integrin β4 is sufficient to severely attenuate breast cancer invasion in a laminin-rich ECM. Furthermore, as NF-κB is a key mediator of integrin β4 intracellular signaling, we demonstrate that inhibition of this pro-survival factor is sufficient to elicit a phenotypic reversion in 3D culture to structures with acinar-like morphology.

While we have implicated mutant p53, integrin β4 and NF-κB in maintenance of the malignant state, there are a number of ways in which to expand upon these findings. One the key ways to confirm that these findings are in fact necessary for the phenotypic effects would be to overexpress integrin β4 to prevent the phenotypic reversion normally observed after mutant p53 depletion. Likewise, one could express a constitutively active version of NF-κB (or a dominant negative version of the NF-κB inhibitor, IκB) to examine whether this can rescue the phenotypic effects of mutant p53 or integrin β4 knockdown. Interestingly, tumor-derived mutants of p53 have already been demonstrated to promote nuclear translocation and activation of NF-κB, although the mechanisms by which mutant p53 elicits this effect have not yet been determined (Weisz et al., 2007). It is tempting to speculate that this earlier observation is due to mutant p53-mediated upregulation of integrin β4, with subsequent NF-κB translocation. In order to test this,

one could examine if inhibition of integrin β4, by RNAi or perhaps by function-blocking antibodies (Gabarra et al., 2010), could prevent the ability of mutant p53 to activate NF-κB.

While we have demonstrated that mutant p53 can interact with two regions of DNA surrounding the *ITGB4* gene using chromatin immunoprecipitation, we cannot yet say by what mechanism mutant p53 is recruited to these sites. It will be very interesting to look for putative transcription factor response elements in the regions with peak mutant p53 binding. Perhaps mutant p53 is recruited to these regions by one of its known interacting partners (NF-Y, Sp1, Ets-1, SREBP or VDR), a novel mutant p53 interacting protein or perhaps it is recruited directly by specific DNA sequences, DNA structures or perhaps histone modifications.

Additionally, since integrin β4 is reported to impinge upon a number of intracellular signaling pathways, it will be very interesting to examine the genome-wide expression changes following depletion of integrin β4 from breast cancer cells grown in 3D culture. Ideally, this will be performed on cell lines engineered with inducible shRNA targeting integrin β4 to exclude cell line differences. Similarly, as we have already implicated NF-κB in this 3D phenotype, it will be very interesting to examine the mRNA expression changes following NF-κB inhibition in breast cancer cells grown in 3D culture. If one were to compare expression changes from these two approaches with expression changes following mutant p53 depletion, one might gain better insight into the target genes necessary for the observed effects on mammary tissue architecture.

Similar to the experiments proposed in relation to mutant p53's ability to upregulate the mevalonate pathway, it will be very interesting to examine which p53 mutants are able to upregulate *ITGB4*. As discussed in Chapter 1, mutant p53 proteins are often classified as "DNA-contact" or "conformational" mutants (Brosh and Rotter, 2009). As these classes have been

shown to have differential affinity toward certain interacting partners, such as p63/p73, perhaps one class may interact preferentially with the SREBP or other transcription factors. While we do not yet known whether mutant p53 is directly recruited to the *ITGB4* promoter or if it is brought there by another other DNA binding protein, it will be interesting to examine whether different mutants have an altered recruitment to this region of DNA.

Improving 3D models of mammary morphogenesis

Much has already been discovered by utilizing three-dimensional culture of mammary epithelial cells in a laminin-rich ECM and this system has been shown to closely model many processes with *in vivo* relevance. While the role of the microenvironment in tumorigenesis is becoming increasing recognized, it is crucial to remember that mammary epithelial cells normally develop in the context of a diverse array of stromal components that may play critical roles in development and carcinogenesis.

One avenue to more faithfully recapitulate the *in vivo* microenvironment is the development of heterotypic 3D culture systems, in which mammary epithelial cells are cultured in combination with other cell types (myoepithelial cells, fibroblasts, endothelial cells, etc.). While carcinogenesis is often simplified to sequential genetic alterations in epithelial cells, tumors do not arise in a vacuum. In the mammary gland, luminal epithelial cells are normally surrounded by myoepithelial cells. As previously described, luminal epithelial cells can develop into acinar-like structures with correct apicobasal polarity and a hollow lumen when cultured in a laminin-rich ECM; however, when these same cells are placed in a collagen-I matrix, they develop into spherical structures that exhibit inverted apicobasal polarity and with no apparent lumina (Gudjonsson et al., 2002). When luminal epithelial cells were co-cultured with primary

myoepithelial cells in a collagen-I environment, the resulting bi-layered structures regained proper apicobasal polarity, a function which was shown to be dependent on laminin-111 expression by myoepithelial cells. Intriguingly, many tumor-associated myoepithelial cells have lost the ability to synthesize laminin-111 and are thus unable to direct polarity in luminal epithelial cells (Gudjonsson et al., 2002). Myoepithelial cells are retained during ductal carcinoma *in situ*, but are often lost at the transition to invasive breast cancer (Holliday et al., 2009). While loss of myoepithelial cells was long thought to be an effect of tumor progression, there is now substantial data suggesting that myoepithelial cells may exert tumor suppressive functions which must be overcome in order to progress to invasive breast cancer. Some of these effects are thought to be paracrine mechanisms as conditioned media from myoepithelial cells has been shown to exert anti-proliferative and anti-invasive effects on a number of breast cancer cells through the secretion of maspin or other factors (Shao et al., 1998). While heterotypic 3D culture models offer much promise to more closely mimic the cellular microenvironment found *in vivo*, many technical limitations limit the use and interpretation of these systems as it is often difficult to determine which cell type is exerting an effect on another. One particularly auspicious avenue might be to develop inter-species heterotypic 3D culture systems, which would allow reliable testing for paracrine effects using species-specific primers/antibodies. Additionally, each cell type can be engineered to express unique fluorescence markers for rapid discrimination and sorting.

Another important aspect of improving 3D culture models of breast cancer is a larger collection of non-malignant mammary epithelial cell lines. As noted above, commonly used cell lines include MCF10A and S1 human mammary epithelial cells, as well as Eph4 and SCp2 murine mammary epithelial cells. While MCF10A cells have been shown to be non-tumorigenic

in xenograft models (Soule et al., 1990), the term "non-malignant" is often preferred over "normal" (although many reports use these cells as "normal" cells). MCF10A cells were derived from a reduction mammoplasty of a patient with fibrocystic changes, and then spontaneously immortalized in culture (Soule et al., 1990). They have been shown to be near-diploid with a stable karyotype; however, they also have amplified *MYC* as well as a deletion of the locus encoding p16 and $p14^{ARF}$. Additionally, they express multiple markers of both luminal and myoepithelial cells, suggesting that this cell line may not perfectly mimic pure mammary epithelial cells *in vivo* (Debnath et al., 2003). That being said, these cells continue to provide valuable insights into mammary development and the effects of cancer-associated genes on tissue architecture. However, a wider array of non-tumorigenic, immortalized mammary epithelial cells will greatly advance our understanding of mammary development and carcinogenesis.

Saudade: The Hunt for a Mutant p53 Response Element(s)

Finally, one of the most fascinating questions in the p53 field may have specific relevance to this project. As mentioned previously, it is often assumed that tumor-derived mutants of p53 completely lack sequence-specificity. It is well documented that at least the hot-spot p53 mutants can no longer recognize canonical wild-type p53 response elements (Bargonetti et al., 1991; Bargonetti et al., 1993; Kern et al., 1992; Thukral et al., 1995). However, as many of these mutations affect residues which bind to DNA, they may in fact acquire novel cognate binding sites, which may be similar or dissimilar to the canonical wild-type p53 response element. This is especially possible for DNA-contact mutants which retain near wild-type conformation of the DNA-binding domain, with one altered amino acid that contacts the DNA backbone.

To our knowledge, a thorough analysis on individual p53 mutants has not been performed to determine whether there is such a thing as a "mutant p53 response element(s)." One of our future plans is to investigate this hypothesis using a combined approach. We first plan to utilize a novel method for Systematic Evolution of Ligands by Exponential Enrichment with concomitant massively parallel sequencing ("SELEX-Seq"). SELEX is a common technique used to identify specific sequences that bind with high-affinity to a target protein and has been successfully applied to wild-type p53 and p63 in the past (Conrad et al., 1996; Fitzwater and Polisky, 1996; Perez et al., 2007; Tuerk and Gold, 1990). However, from traditional SELEX, one only identifies the sequences that bind with highest affinity. With the addition of massively parallel sequencing between each round of SELEX, one can use a novel algorithm developed in the Bussemaker laboratory (Harmen Bussemaker and Todd Riley, *personal communication*) to more accurately infer nucleotide preferences at a given position. This allows one to see relative preferences at each site, which may be much more important when investigating DNA binding by neomorphic p53 proteins. We plan to search for sequences which bind to a number of frequently occurring p53 mutants (p53-R175H, -G245S, -R248Q, -R248W, -R273H, -R280K), and we plan to include wild-type p53 as a control for the technique. This may provide novel information regarding specific sequence motifs that might serve as "mutant p53 response elements," in addition to deepening our understanding about the mechanisms by which wild-type p53 recognizes DNA elements. The second part of the approach will be to confirm any sequence elements identified as potential mutant p53 binding sequences. We plan to search for this site(s) in our mutant p53 ChIP-Seq to examine whether these are enriched over the control samples as well as perform electrophoretic mobility shift assays (EMSA), mutating key nucleotides, to validate this binding.

Concluding Remarks

Overall in this dissertation, we describe a novel role for tumor-derived p53 mutants in the disruption of mammary tissue architecture. Using the three-dimensional (3D) ECM culture model, in which non-malignant mammary epithelial cells form structures highly reminiscent for many features of acini *in vivo*, while breast cancer cells for disorganized, malignant-appearing structures, we were able to uncover novel functions of mutant forms of p53. These include upregulation of multiple genes associated with the mevalonate pathway, as well as a key integrin responsible for cell-ECM communication. Interestingly, both of these have been implicated in tumorigenesis, although not previously connected to mutant p53. This work not only contributes to our understanding of breast carcinogenesis, but may also provide potentially valuable prognostic tools and therapeutic targets for mutant p53 tumors.

REFERENCES

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V.*, et al.* (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell *137*, 87-98.

Ahern, T.P., Pedersen, L., Tarp, M., Cronin-Fenton, D.P., Garne, J.P., Silliman, R.A., Sorensen, H.T., and Lash, T.L. (2011). Statin Prescriptions and Breast Cancer Recurrence Risk: A Danish Nationwide Prospective Cohort Study. J Natl Cancer Inst.

Alsner, J., Jensen, V., Kyndi, M., Offersen, B.V., Vu, P., Borresen-Dale, A.L., and Overgaard, J. (2008). A comparison between p53 accumulation determined by immunohistochemistry and TP53 mutations as prognostic variables in tumours from breast cancer patients. Acta Oncol *47*, 600-607.

Bargonetti, J., Chicas, A., White, D., and Prives, C. (1997). p53 represses Sp1 DNA binding and HIV-LTR directed transcription. Cell Mol Biol (Noisy-le-grand) *43*, 935-949.

Bargonetti, J., Friedman, P.N., Kern, S.E., Vogelstein, B., and Prives, C. (1991). Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. Cell *65*, 1083-1091.

Bargonetti, J., Manfredi, J.J., Chen, X., Marshak, D.R., and Prives, C. (1993). A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. Genes Dev *7*, 2565-2574.

Barsotti, A.M., and Prives, C. (2010). Noncoding RNAs: the missing "linc" in p53-mediated repression. Cell *142*, 358-360.

Bartek, J., Iggo, R., Gannon, J., and Lane, D.P. (1990). Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene *5*, 893-899.

Beliveau, A., Mott, J.D., Lo, A., Chen, E.I., Koller, A.A., Yaswen, P., Muschler, J., and Bissell, M.J. (2010). Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. Genes Dev *24*, 2800-2811.

Bissell, M.J., Kenny, P.A., and Radisky, D.C. (2005). Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes. Cold Spring Harb Symp Quant Biol *70*, 343-356.

Bossi, G., Lapi, E., Strano, S., Rinaldo, C., Blandino, G., and Sacchi, A. (2006). Mutant p53 gain of function: reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression. Oncogene *25*, 304-309.

Botcheva, K., McCorkle, S.R., McCombie, W.R., Dunn, J.J., and Anderson, C.W. (2011). Distinct p53 genomic binding patterns in normal and cancer-derived human cells. Cell Cycle *10*.

Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer *9*, 701-713.

Chicas, A., Molina, P., and Bargonetti, J. (2000). Mutant p53 forms a complex with Sp1 on HIV-LTR DNA. Biochem Biophys Res Commun *279*, 383-390.

Chrisanthar, R., Knappskog, S., Lokkevik, E., Anker, G., Ostenstad, B., Lundgren, S., Risberg, T., Mjaaland, I., Skjonsberg, G., Aas, T.*, et al.* (2011). Predictive and prognostic impact of TP53 mutations and MDM2 promoter genotype in primary breast cancer patients treated with epirubicin or paclitaxel. PLoS One *6*, e19249.

Conrad, R.C., Giver, L., Tian, Y., and Ellington, A.D. (1996). In vitro selection of nucleic acid aptamers that bind proteins. Methods Enzymol *267*, 336-367.

Davison, T.S., Yin, P., Nie, E., Kay, C., and Arrowsmith, C.H. (1998). Characterization of the oligomerization defects of two p53 mutants found in families with Li-Fraumeni and Li-Fraumeni-like syndrome. Oncogene *17*, 651-656.

Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods *30*, 256-268.

Di Agostino, S., Strano, S., Emiliozzi, V., Zerbini, V., Mottolese, M., Sacchi, A., Blandino, G., and Piaggio, G. (2006). Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer Cell *10*, 191-202.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

Donehower, L.A., and Lozano, G. (2009). 20 years studying p53 functions in genetically engineered mice. Nat Rev Cancer *9*, 831-841.

Fagherazzi, G., Fabre, A., Boutron-Ruault, M.C., and Clavel-Chapelon, F. (2010). Serum cholesterol level, use of a cholesterol-lowering drug, and breast cancer: results from the prospective E3N cohort. Eur J Cancer Prev *19*, 120-125.

Fitzwater, T., and Polisky, B. (1996). A SELEX primer. Methods Enzymol *267*, 275-301.

Freed-Pastor, W.A.P., C. (2011). Dissimilar DNA Binding by p53 in Normal and Tumor-derived Cells. Cell Cycle.

Friedman, S.L., Shaulian, E., Littlewood, T., Resnitzky, D., and Oren, M. (1997). Resistance to p53-mediated growth arrest and apoptosis in Hep 3B hepatoma cells. Oncogene *15*, 63-70.

Gabarra, V., Cho, S., Ramirez, M., Ren, Y., Chen, L.L., Cheung, A., Cao, X., Rennard, R., Unruh, K.R., Graff, C.P.*, et al.* (2010). Antibodies directed to alpha6beta4 highlight the adhesive and signaling functions of the integrin in breast cancer cell lines. Cancer Biol Ther *9*, 437-445.

Girardini, J.E., Napoli, M., Piazza, S., Rustighi, A., Marotta, C., Radaelli, E., Capaci, V., Jordan, L., Quinlan, P., Thompson, A.*, et al.* (2011). A Pin1/mutant p53 axis promotes aggressiveness in breast cancer. Cancer Cell *20*, 79-91.

Gudjonsson, T., Ronnov-Jessen, L., Villadsen, R., Rank, F., Bissell, M.J., and Petersen, O.W. (2002). Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. J Cell Sci *115*, 39-50.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Holliday, D.L., Brouilette, K.T., Markert, A., Gordon, L.A., and Jones, J.L. (2009). Novel multicellular organotypic models of normal and malignant breast: tools for dissecting the role of the microenvironment in breast cancer progression. Breast Cancer Res *11*, R3.

Kern, S.E., Pietenpol, J.A., Thiagalingam, S., Seymour, A., Kinzler, K.W., and Vogelstein, B. (1992). Oncogenic forms of p53 inhibit p53-regulated gene expression. Science *256*, 827-830.

Lang, G.A., Iwakuma, T., Suh, Y.A., Liu, G., Rao, V.A., Parant, J.M., Valentin-Vega, Y.A., Terzian, T., Caldwell, L.C., Strong, L.C.*, et al.* (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell *119*, 861-872.

Liang, S.H., and Clarke, M.F. (1999). A bipartite nuclear localization signal is required for p53 nuclear import regulated by a carboxyl-terminal domain. J Biol Chem *274*, 32699-32703.

Lin, J., Chen, J., Elenbaas, B., and Levine, A.J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes Dev *8*, 1235-1246.

Liu, G., McDonnell, T.J., Montes de Oca Luna, R., Kapoor, M., Mims, B., El-Naggar, A.K., and Lozano, G. (2000). High metastatic potential in mice inheriting a targeted p53 missense mutation. Proc Natl Acad Sci U S A *97*, 4174-4179.

Lumachi, F., Marino, F., Varotto, S., and Basso, U. (2009). Oligonucleotide probe array for p53 gene alteration analysis in DNA from formalin-fixed paraffin-embedded breast cancer tissues. Ann N Y Acad Sci *1175*, 89-92.

Mizuno, H., Spike, B.T., Wahl, G.M., and Levine, A.J. (2010). Inactivation of p53 in breast cancers correlates with stem cell transcriptional signatures. Proc Natl Acad Sci U S A *107*, 22745-22750.

Moll, U.M., Riou, G., and Levine, A.J. (1992). Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. Proc Natl Acad Sci U S A *89*, 7262-7266.

Muller, P.A., Caswell, P.T., Doyle, B., Iwanicki, M.P., Tan, E.H., Karim, S., Lukashchuk, N., Gillespie, D.A., Ludwig, R.L., Gosselin, P., *et al.* (2009). Mutant p53 drives invasion by promoting integrin recycling. Cell *139*, 1327-1341.

Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell *119*, 847-860.

Oren, M., and Rotter, V. (2010). Mutant p53 gain-of-function in cancer. Cold Spring Harb Perspect Biol *2*, a001107.

Peart, M.J., and Prives, C. (2006). Mutant p53 gain of function: the NF-Y connection. Cancer Cell *10*, 173-174.

Perez, C.A., Ott, J., Mays, D.J., and Pietenpol, J.A. (2007). p63 consensus DNA-binding site: identification, analysis and application into a p63MH algorithm. Oncogene *26*, 7363-7370.

Reed, B.D., Charos, A.E., Szekely, A.M., Weissman, S.M., and Snyder, M. (2008). Genome-wide occupancy of SREBP1 and its partners NFY and SP1 reveals novel functional roles and combinatorial regulation of distinct classes of genes. PLoS Genet *4*, e1000133.

Rodriguez-Cruz, M., Tovar, A.R., Palacios-Gonzalez, B., Del Prado, M., and Torres, N. (2006). Synthesis of long-chain polyunsaturated fatty acids in lactating mammary gland: role of Delta5 and Delta6 desaturases, SREBP-1, PPARalpha, and PGC-1. J Lipid Res *47*, 553-560.

Rudolph, M.C., McManaman, J.L., Phang, T., Russell, T., Kominsky, D.J., Serkova, N.J., Stein, T., Anderson, S.M., and Neville, M.C. (2007). Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine. Physiol Genomics *28*, 323-336.

Russo, J., and Russo, I.H. (1996). Experimentally induced mammary tumors in rats. Breast Cancer Res Treat *39*, 7-20.

Sabbisetti, V., Di Napoli, A., Seeley, A., Amato, A.M., O'Regan, E., Ghebremichael, M., Loda, M., and Signoretti, S. (2009). p63 promotes cell survival through fatty acid synthase. PLoS One *4*, e5877.

Sampath, J., Sun, D., Kidd, V.J., Grenet, J., Gandhi, A., Shapiro, L.H., Wang, Q., Zambetti, G.P., and Schuetz, J.D. (2001). Mutant p53 cooperates with ETS and selectively up-regulates human MDR1 not MRP1. J Biol Chem *276*, 39359-39367.

Sarig, R., Rivlin, N., Brosh, R., Bornstein, C., Kamer, I., Ezra, O., Molchadsky, A., Goldfinger, N., Brenner, O., and Rotter, V. (2010). Mutant p53 facilitates somatic cell reprogramming and augments the malignant potential of reprogrammed cells. J Exp Med *207*, 2127-2140.

Seo, Y.K., Chong, H.K., Infante, A.M., Im, S.S., Xie, X., and Osborne, T.F. (2009). Genome-wide analysis of SREBP-1 binding in mouse liver chromatin reveals a preference for promoter proximal binding to a new motif. Proc Natl Acad Sci U S A *106*, 13765-13769.

Seo, Y.K., Jeon, T.I., Chong, H.K., Biesinger, J., Xie, X., and Osborne, T.F. (2011). Genome-wide localization of SREBP-2 in hepatic chromatin predicts a role in autophagy. Cell Metab *13*, 367-375.

Shao, Z.M., Nguyen, M., Alpaugh, M.L., O'Connell, J.T., and Barsky, S.H. (1998). The human myoepithelial cell exerts antiproliferative effects on breast carcinoma cells characterized by p21WAF1/CIP1 induction, G2/M arrest, and apoptosis. Exp Cell Res *241*, 394-403.

Smeenk, L., van Heeringen, S.J., Koeppel, M., Gilbert, B., Janssen-Megens, E., Stunnenberg, H.G., and Lohrum, M. (2011). Role of p53 serine 46 in p53 target gene regulation. PLoS One *6*, e17574.

Song, H., Hollstein, M., and Xu, Y. (2007). p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. Nat Cell Biol *9*, 573-580.

Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, W.D., Jr., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F., and Brooks, S.C. (1990). Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res *50*, 6075-6086.

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Siegfried, Z., Shiff, I., Kogan, I., Shay, M., Kalo, E.*, et al.* (2010). Modulation of the vitamin D3 response by cancer-associated mutant p53. Cancer Cell *17*, 273-285.

Stommel, J.M., Marchenko, N.D., Jimenez, G.S., Moll, U.M., Hope, T.J., and Wahl, G.M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. EMBO J *18*, 1660-1672.

Sukumar, S., McKenzie, K., and Chen, Y. (1995). Animal models for breast cancer. Mutat Res *333*, 37-44.

Sun, X.X., Dai, M.S., and Lu, H. (2007). 5-fluorouracil activation of p53 involves an MDM2-ribosomal protein interaction. J Biol Chem *282*, 8052-8059.

Terzian, T., Suh, Y.A., Iwakuma, T., Post, S.M., Neumann, M., Lang, G.A., Van Pelt, C.S., and Lozano, G. (2008). The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. Genes Dev *22*, 1337-1344.

Thukral, S.K., Lu, Y., Blain, G.C., Harvey, T.S., and Jacobsen, V.L. (1995). Discrimination of DNA binding sites by mutant p53 proteins. Mol Cell Biol *15*, 5196-5202.

Tuerk, C., and Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science *249*, 505-510.

Urist, M., Tanaka, T., Poyurovsky, M.V., and Prives, C. (2004). p73 induction after DNA damage is regulated by checkpoint kinases Chk1 and Chk2. Genes Dev *18*, 3041-3054.

Wang, F., Weaver, V.M., Petersen, O.W., Larabell, C.A., Dedhar, S., Briand, P., Lupu, R., and Bissell, M.J. (1998). Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc Natl Acad Sci U S A *95*, 14821-14826.

Weaver, V.M., Petersen, O.W., Wang, F., Larabell, C.A., Briand, P., Damsky, C., and Bissell, M.J. (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol *137*, 231-245.

Wei, C.L., Wu, Q., Vega, V.B., Chiu, K.P., Ng, P., Zhang, T., Shahab, A., Yong, H.C., Fu, Y., Weng, Z.*, et al.* (2006). A global map of p53 transcription-factor binding sites in the human genome. Cell *124*, 207-219.

Weisz, L., Damalas, A., Liontos, M., Karakaidos, P., Fontemaggi, G., Maor-Aloni, R., Kalis, M., Levrero, M., Strano, S., Gorgoulis, V.G.*, et al.* (2007). Mutant p53 enhances nuclear factor kappaB activation by tumor necrosis factor alpha in cancer cells. Cancer Res *67*, 2396-2401.

Xu, J., Reumers, J., Couceiro, J.R., De Smet, F., Gallardo, R., Rudyak, S., Cornelis, A., Rozenski, J., Zwolinska, A., Marine, J.C.*, et al.* (2011). Gain of function of mutant p53 by coaggregation with multiple tumor suppressors. Nat Chem Biol *7*, 285-295.

Yan, W., and Chen, X. (2010). Characterization of functional domains necessary for mutant p53 gain of function. J Biol Chem *285*, 14229-14238.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W.*, et al.* (2008). Model-based analysis of ChIP-Seq (MACS). Genome Biol *9*, R137.

Zhu, J., Zhou, W., Jiang, J., and Chen, X. (1998). Identification of a novel p53 functional domain that is necessary for mediating apoptosis. J Biol Chem *273*, 13030-13036.

FIGURE LEGENDS

Figure 4.1 shRNA-resistant mutant p53 can rescue the phenotypic reversion observed after depletion of endogenous mutant p53

MDA-468.shp53 were engineered to express shRNA-resistant versions of tumor-derived p53 mutants and assessed for their morphology in 3D culture. Cells were grown in 3D cultures for 5 days in the presence or absence of DOX as indicated. Representative differential interference contrast (DIC) microscopy is shown for (A) MDA-468.shp53, (B) MDA-468.shp53/pLNCX-Empty, (C) MDA-468.shp53/Flag-p53-R175H, (D) MDA-468.shp53/Flag-p53-R175H-mTAD, (E) MDA-468.shp53/Flag-p53-R273H, (F) MDA-468.shp53/Flag-p53-R273H-mTAD, (G) MDA-468.shp53/Flag-p53-R248W, (H) MDA-468.shp53/Flag-p53-R248W-mTAD, (I) MDA-468.shp53/Flag-p53-G245S, (J) MDA-468.shp53/Flag-p53-G245S-mTAD.

Figure 4.2 Sterol biosynthesis genes are significantly enriched in a mutant p53 ChIP-Seq from MDA-468.shp53 cells grown in 3D culture

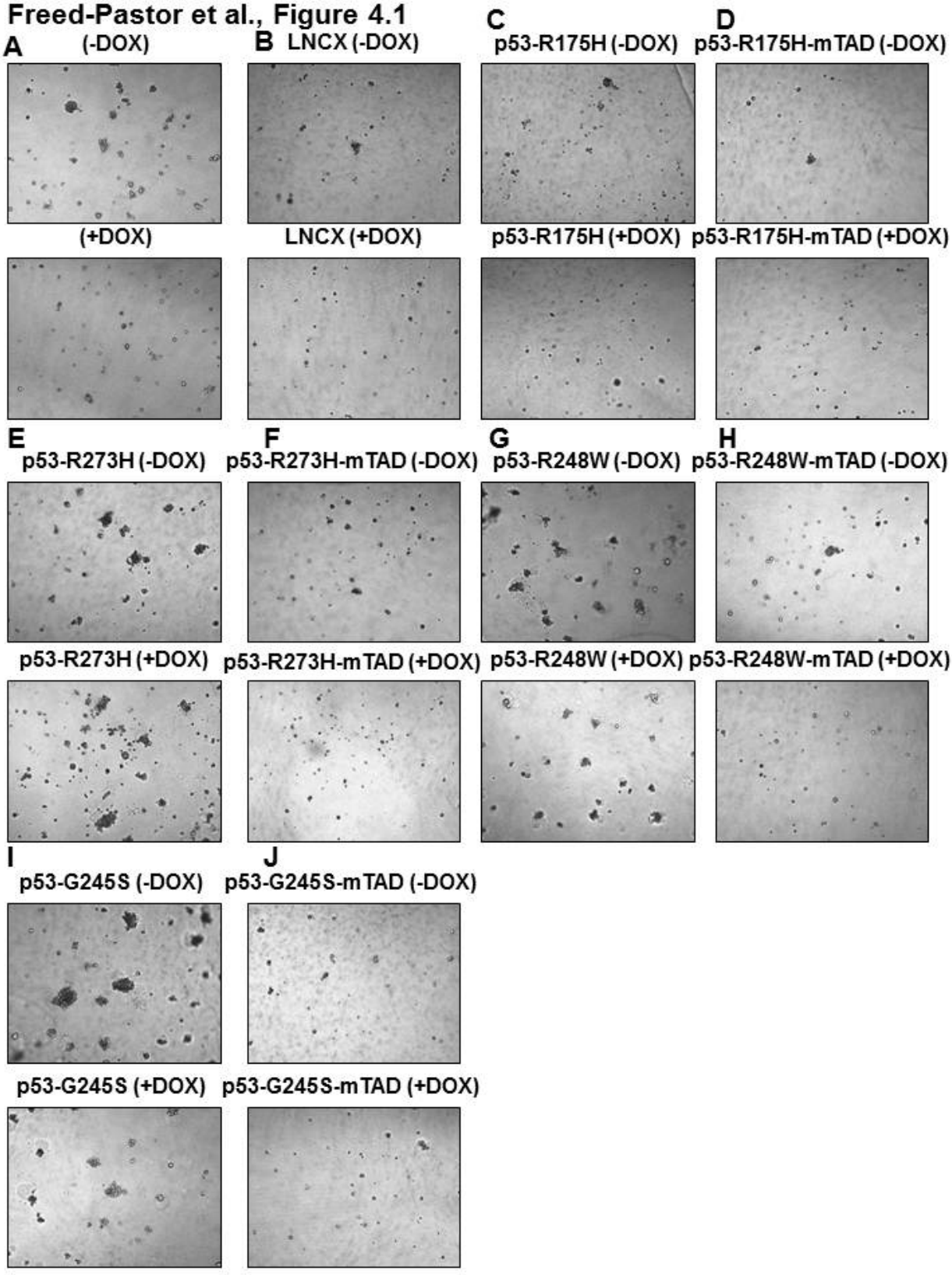
MDA-468.shp53 cells were grown in 3D culture for 8 days in the presence or absence of DOX to deplete cells of p53. Samples were crosslinked with 1% formaldehyde, sonicated and mutant p53 was immunoprecipitated with anti-p53 antibodies (DO-1 or 421) or control mouse IgG. DNA was then subjected to Illumina sequencing. Reads were mapped to the human genome using Bowtie and peaks were identified using MACS (Zhang et al., 2008). After assigning peaks to annotated genes based on the criteria: p-value $\leq 10^{-6}$ and fold enrichment ≥ 5 compared to IgG control or "+DOX," the relative enrichment of the sterol biosynthesis genes (Figure 2.S3) or

SREBP1 ChIP-on-chip target genes (Reed et al., 2008) was assessed. P-values are based on Fisher's exact test.

Figure 4.3 Wild-type p53 can repress the mevalonate pathway genes

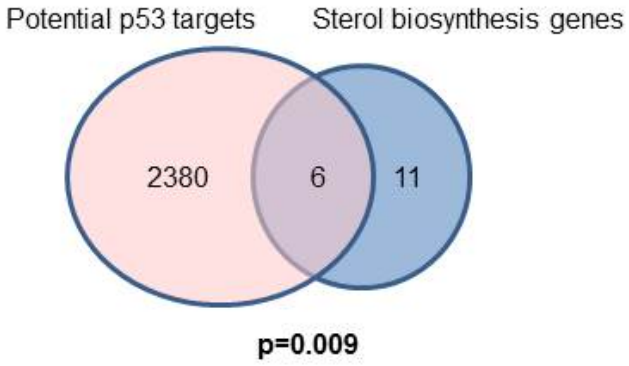
(A) SK-Hep1 cells were treated for 24 hours with DMSO control, Nutlin-3a (10 μM) or 5-fluorouracil (5-FU) (0.5 μM). Cells were harvested and the mRNA expression of the specified sterol biosynthesis genes was assessed by qRT-PCR. A representative experiment is shown.

(B) Hep3B-4Bv cells, expressing a temperature-sensitive version of p53, were cultured at either 37^{o}C (mutant p53 conformation) or 32^{o}C (wild-type p53 conformation) for 48 hours. Cells were harvested and the mRNA expression of the specified sterol biosynthesis genes was assessed by qRT-PCR. Data presented as mean ± st dev of three independent experiments. *indicates $p<0.05$, **indicates $p<0.01$ from a two-sided t-test.

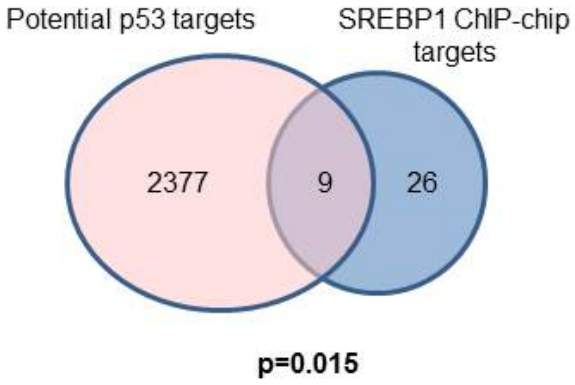

Freed-Pastor et al., Figure 4.1
A
(-DOX)
B
LNCX (-DOX)
C
p53-R175H (-DOX)
D
p53-R175H-mTAD (-DOX)
(+DOX)
LNCX (+DOX)
p53-R175H (+DOX)
p53-R175H-mTAD (+DOX)
E
p53-R273H (-DOX)
F
p53-R273H-mTAD (-DOX)
G
p53-R248W (-DOX)
H
p53-R248W-mTAD (-DOX)
p53-R273H (+DOX)
p53-R273H-mTAD (+DOX)
p53-R248W (+DOX)
p53-R248W-mTAD (+DOX)
I
p53-G245S (-DOX)
J
p53-G245S-mTAD (-DOX)
p53-G245S (+DOX)
p53-G245S-mTAD (+DOX)

Freed-Pastor et al., Figure 4.2

A

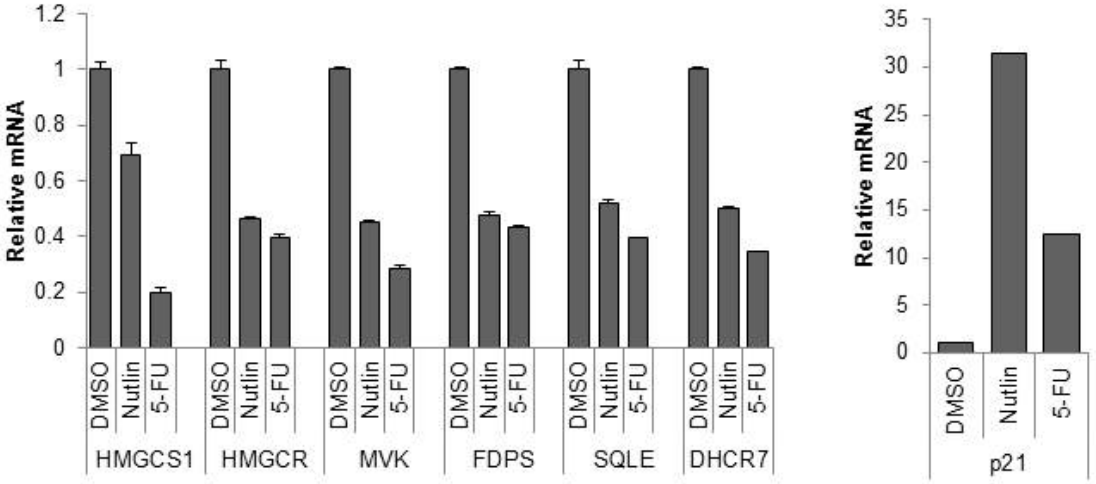


B



Freed-Pastor et al., Figure 4.3

A



B